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VOLUME 7

GRAS MONOGRAPH SERIES

ASCORBIC ACID

(COPIES OF ARTICLES CITED BUT NOT USED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE

OCTOBER 7, 1974

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Tracor Jitco, Inc.

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This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

J. Chem. Education 11, 69-72 (1934)

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UNLOCKING ANOTHER DOOR *to* NATURE'S SECRETS—VITAMIN C

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This paper is a review dealing with the recent isolation and identification of vitamin C. The chemical properties of the vitamin are described, and the methods of assaying it and its physiological effects are given. The formulas that have been suggested for the constitution of the vitamin are reported and the natural sources of the antiscorbutic substance described.

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THE NUTRITIONAL requirements of the higher animals and man have been found in recent years to be very complex, but along with the recognition of complexity has come steady progress in identifying the newer factors, making possible a clearer picture of physiological processes.

In addition to fats, proteins, carbohydrates, water, and inorganic salts, a number of accessory factors known as vitamins are necessary for the maintenance of health. The hormones and enzymes, which resemble the vitamins in that they are organic compounds possessing remarkable activity, are physiologically in a different class because they are produced by the body. The vitamins must be obtained from some outside source. Therefore, their supply to the body is largely a matter of chance, and it can easily be understood that prolonged deficiencies in specific factors may occur on restricted or highly artificial diets. The results—such conditions as rickets, pellagra, and scurvy—have plagued the human race. Although scurvy, which is caused by a prolonged lack of vitamin C, is no longer of frequent occurrence in its most severe form, occasional outbreaks still occur, and there is good evidence that partial vitamin C deficiency is a factor to be considered seriously in relation to public health.

As early as 1593 Albertus noticed a disease in men which was characterized by loss in weight, weakness, pallor, sore and swollen joints, and the falling out of teeth, but apparently he did not clearly recognize that it was the result of a deficiency. It is interesting to note, however, that he was able to cure the disease by the administration of sour juices. Much later *(1720)* Kramer recognized scurvy as a deficiency disease, and Lind *(1)* (1752) confirmed this view by a series of experiments on twelve human subjects under "experimental control."

That the cause of scurvy was a deficiency of some unidentified organic substance in citrus fruits and vegetables was clearly stated by Budd *(2)* in 18[illegible]. The discovery by Holst and Frölich *(3)* in 1907 that a disease analogous to human scurvy could be produced in guinea pigs by dietary control laid the basis for most of the modern investigations and progress.

CHEMICAL PROPERTIES

Information concerning the chemical properties of vitamin C was accumulating, but necessarily remained somewhat empirical previous to its isolation and identification (King and Waugh *(4)*). Bezssonov *(5)*, who suggested that the vitamin consisted of three factors, a sugar, an organic acid, and a phenol, had made many contributions to the knowledge of behavior of the vitamin in his attempts at isolation. Zilva *(6)* and associates had made highly active preparations and had contributed a great deal to our knowledge of the vitamin during several years of laboratory study. His finding that after the vitamin had been destroyed by atmospheric oxidation it could not be reduced with hydrogen and platinum black *(7)*, and his methods of concentration proved of particular value. Sherman *(8)* and associates demonstrated clearly the effects of the pH of its solutions upon its rate of destruction, and improved the method of assay. Hess and associates *(9)* had made many contributions to its clinical significance, and had shown that copper and other heavy metals catalyze the oxidation of the antiscorbutic factor.

McKinnis and King *(10)* showed that the active factor diffuses rapidly through a collodion membrane (demonstrated by feeding tests on the dialysate), and from their electrical transference studies at different pH values found that the vitamin was not a salt-forming nitrogen compound, such as an amino acid or base, but possessed distinct acidic properties. Sipple, Grettie, and Svirbely, in collaboration with Dr. King *(11)*, developed improved methods of concentrating the active factor and showed that it is soluble in absolute acetone, ethyl acetate, and propyl alcohol, but insoluble in ethyl ether and petroleum ether. Smith and King *(12)* showed that purified concentrates free from enzymes and heavy metals, can be stored for fourteen days or more without appreciable loss of activity. The reducing value, solubility, sirupy nature, and acidity of this concentrate corresponded with the properties of an active hexuronic acid.

Very early in 1932 Rygh, Rygh, and Laland (*13*) published their evidence for having isolated and synthesized vitamin C. A compound derived from narcotine was reported to have protected guinea pigs from scurvy (but survival was not beyond that of negative controls). King and Waugh (*4*) pointed out that their technic led to a misinterpretation of their results in that the only animals surviving the experimental period had received the vitamin from boiled orange juice or an aqueous extract of sprouted grain. Many investigators [Zilva, Ott, Harris, and Tillmans (*14*)] have been unable to duplicate Rygh's results.

As a well-identified substance, the former mysterious nutritive factor, measured only in terms of 8- to 12-week animal tests, is now studied as a simple organic compound.

METHODS OF ASSAY

At the present time there are two general methods for the determination of vitamin C, a chemical method and the physiological assay, the latter being essentially the criterion which must still be used to substantiate results by the chemical method (which is not specific for the vitamin).

The chemical method was developed principally by Tillmans and associates (*15*) who pointed out the striking correlation between the reducing value of foods and their vitamin C content. It depends on the reduction of a dye, 2,6-dichlorophenolindophenol (introduced in the series of oxidation-reduction indicators synthesized in Mansfield Clark's laboratory) by the vitamin. The method is fairly accurate for determining the amount of vitamin C in both animal and plant sources when there is no other strong reducing material present. Such compounds (*e. g.*, cysteine) are known to occur, however, and hence the test can only be used with reservation.

The standard physiological method depends on the use of guinea pigs; these animals are very sensitive to a deficiency of the antiscorbutic material and can be relied upon to manifest reasonably well-defined symptoms. The method most generally used is the one proposed by Sherman, La Mer, and Campbell (*16*). The animals are given a basal diet which contains everything for normal growth except vitamin C. When given this diet alone, the animals will die within a period of twenty-five to thirty-five days. When a food or preparation is to be assayed, it is fed in graded amounts so that the minimum protective level is demonstrated on a quantitative basis. A tooth-cross-section technic with guinea pigs, and a curative test have been used in several laboratories successfully.

ISOLATION AND IDENTIFICATION

Studying oxidation-reduction factors in Professor Hopkins' laboratory at Cambridge University in 1928, Szent-Györgyi (*17*) isolated from cabbages, oranges, and adrenal glands a very active reducing substance which he termed "hexuronic acid." Certain of Zilva's results (*20*) indicated that the acid could not be directly related to vitamin C, and Szent-Györgyi sought to relate it to cortin, the adrenal hormone (*18*).

In the early fall of 1931, after four years of systematic fractionation and assay of the antiscorbutic material from lemon juice, crystals which exhibited the properties of Szent-Györgyi's "hexuronic acid" were isolated at the University of Pittsburgh. These crystals showed a minimum protective level of 0.5 mg. per day for guinea pigs under standard assay conditions. This was not reported until March, 1932 (*4*), when further evidence had been obtained, including preparation of the lead salt for alcoholic solution and recrystallization from different solvents, without change in activity, particularly because of Rygh's report of a substance about 10,000 times more active.

The proof that vitamin C was a six-carbon sugar acid (hexuronic acid) and identical with the acid isolated by Szent-Györgyi as a reducing factor in animal and plant tissues may be summarized as follows: (*a*) the acid titration equivalent corresponded with the formula $C_6H_8O_6$; (*b*) two atoms of iodine (or equivalent of other oxidizing agents) were reduced by each mol of acid; (*c*) the optical rotation found was $[\alpha]^{20}_{D} = +24°$ to 25°; (*d*) typical crystals were obtained repeatedly from different solvents; (*e*) the solubility in a number of organic solvents corresponded with such a formula; (*f*) precipitation as a lead salt from an alcohol or water solution was consistent with regard to activity and properties; (*g*) there was a similar instability toward alkaline reagents and oxidizing agents; (*h*) the diffusion rate and electrical transference were typical for such an acid; (*i*) the melting point varied some with different preparations (approximately 185°) and was accompanied by decomposition.

A fortnight later, Szent-Györgyi and Svirbely (on an International Exchange Fellowship from the University of Pittsburgh) (*19*) reported that one milligram of hexuronic acid fed daily protected guinea pigs from scurvy.

The acid prepared from adrenal glands by an entirely different method was generously supplied by E. C. Kendall, and found to exhibit (*27*) the same quantitative physiological response as found for the acid prepared from lemon juice, thus eliminating the possibility of antiscorbutic activity being due to contaminating material. An improved method of preparing the vitamin from lemon juice was reported by Bessey, Waugh, and King (*35*).

Szent-Györgyi and Svirbely (*28*) reported feeding tests with the acetone derivative and also with the acid after hydrolysis from the acetone derivative (reported melting point, 192°), finding it protective on a 0.5 mg. level. In the same paper, they also described a procedure for separating the vitamin from Hungarian red peppers, recovering nearly a pound of the pure vitamin. The preparation of the acetone derivative was described by von Vargha (*29*).

Evidence has rapidly accumulated in a number of laboratories confirming beyond question the identity

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of vitamin C as a six-carbon sugar acid having the general formula $C_6H_8O_6$, for which the name "ascorbic acid" has been suggested by Szent-Györgyi and Haworth. Harris (*14, 26*), Tillmans (*14, 15*), Nelson (*21*), Zilva (*20*), and their associates have made important contributions in confirming the identity of the vitamin.

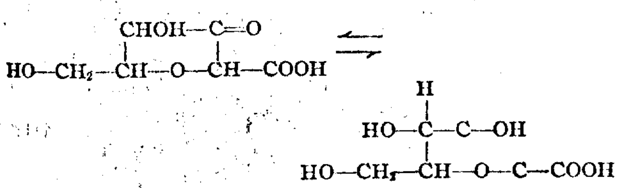
A series of different structural formulas for the acid have been suggested from different laboratories, each laboratory proposing a different formula, so far without adequate proof or verification by animal tests. Cox, Hirst, and Reynolds (*22*) have suggested the formulas:

$$COOH \cdot CO \cdot CO \cdot CH_2 \cdot CHOH \cdot CH_2OH \rightleftarrows COOH \cdot CO \cdot C(OH){:}CH \cdot CHOH \cdot CH_2OH$$

Karrer and associates (*23*) have suggested the formula

$$CH_2OH \cdot CHOH \cdot CO \cdot CH_2 \cdot CO \cdot COOH.$$

The third formula was advanced by Micheel and Kraft (*24*), who suggested the furan-ring type of structure:



Levene and Raymond (*36*) have suggested:

$$\overbrace{CH_2 \cdot CHOH \cdot CHOH \cdot CH}^{O} \cdot CO \cdot COOH$$

as an alternative to the Euler formula (*37*),

$$CH_2OH \cdot CHOH \cdot \overbrace{CH \cdot COH{:}COH \cdot CO}^{O}$$

The final proof of structure will depend on the synthesis of a compound which will function as the vitamin physiologically when fed to guinea pigs, in addition to having identical physical and chemical properties. It is very likely that synthesis will be accomplished in the near future.

Reichstein, Grussner, and Oppenauer* (*25*) have recently reported the synthesis of the *d*-form of vitamin C. They started with *d*-xylosone and obtained a very active reducing material which gave the right melting point but they could not obtain the correct optical rotation. No mention was made of animal feeding tests.

OCCURRENCE AND FUNCTIONS

Vitamin C occurs very widely in the plant kingdom and occasionally in high concentration. Rich plant sources are red and green peppers, lemons, oranges, tomatoes, and green vegetables. It appears probable that it plays a functional rôle in the metabolism of all of the higher plants and animals. In animals the vitamin is highly concentrated in the cortex of the adrenal gland, but is present in other tissues in widely varying amounts. Harris and co-workers (*30*) have published a modification of Tillman's method for the determination of vitamin C in animal tissues.

Largely from Szent-Györgyi's work, before his "hexuronic acid" was identified as the vitamin, it is evident that the vitamin is related to normal respiration. Hartman (*31*) has made the interesting observation that a cortical extract containing cortin but free from hexuronic acid, enabled guinea pigs to retain their weight better on a "C-free" diet. Szule (*32*) has reported that a vitamin C treatment of Addison's disease causes depigmentation.

Birch and Dann (*33*) found that tissues having a high glutathione content also contain a large amount of vitamin C. They suggest that these two substances might be linked factors in one system of oxidation in the animal cell.

Marine and Baumann (*34*) have recently suggested that vitamin C may have some relationship to goiter. Hanke (*37*) has particularly emphasized the practical relation of vitamin C to tooth structure, although such an intimate relation has been recognized by research workers for several years.

* Since this paper went to print these authors have reported [*Helv. Chim. Acta*, **16**, 1019 (1933)] the synthesis of the *l*-form which was physiologically active when fed to guinea pigs.

LITERATURE CITED

(1) LIND, J., "A treatise on scurvy," London, **1757**.
(2) BUDD, C., "Tweedie's system of practice of medicine," Lea and Blanchard, Philadelphia, **1841**.
(3) HOLST, A. AND FRÖLICH, T., "Über experimentellen Skorbut," *Z. Hyg. Infektionskrankh.*, **75**, 334–44 (1913).
(4) KING, C. G AND WAUGH, W. A., "Isolation and identification of vitamin C," *Science*, **75**, 357 (April 1, 1932); WAUGH, W. A. AND KING, C. G., "Isolation and identification of vitamin C," *J. Biol. Chem.*, **97**, 325 (1932).
(5) BEZSSONOV, N., "Color reactions of antiscorbutic extracts with phosphomolybdotungstic acid," *Bull soc. chim. biol.*, **4**, 83–95 (1922).
(6) ZILVA, S. S., "Concentration and potency of concentrated antiscorbutic extracts," *Biochem. J.*, **17**, 410–5 (1923); "Antiscorbutic fractions of lemon juice. VIII," *ibid.*, **22**, 779–85 (1928).
(7) DAUBNEY, C. G. AND ZILVA, S. S., "The action of reducing agents on the antiscorbutic factor inactivated by aëration," *ibid.*, **20**, 519–23 (1926).
(8) LA MER, V. K., CAMPBELL, H. L., AND SHERMAN, H. C., "The effect of temperature and concentration of the H ions upon the rate of destruction of the antiscorbutic vitamin," *J. Am. Chem. Soc.*, **44**, 172–81 (1922).
(9) HESS, A. F., "The antiscorbutic vitamin," *Ind. Eng. Chem.*, **22**, 1015–7 (1930); "Scurvy, past and present," J. B. Lippincott, Philadelphia, **1915**.
(10) MCKINNIS, R. B. AND KING, C. G., "The nature of vitamin C—a study of electrical transference," *J. Biol. Chem.*, **87**, 615–23 (1930).
(11) SIPPLE, H. L. AND KING, C. G., "A study of the preparation and properties of vitamin C fractions from lemon juice," *J. Am. Chem. Soc.*, **52**, 420–3 (1930); GRETTIE, D. P. AND KING, C. G., "The preparation and properties of vitamin C fractions from lemon juice," *J. Biol. Chem.*, **84**, 771–6 (1930); SVIRBELY, J. L. AND KING, C. G., "The preparation and properties of vitamin C concentrates from lemon juice," *ibid.*, **94**, 483–8 (1931–32).
(12) SMITH, F. L., II, AND KING, C. G., "The preparation and storage of vitamin C concentrates from lemon juice," *ibid.*, **94**, 491–6 (1931).

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(13) RYGH, O., RYGH, A., AND LALAND, P., "Chemical investigation on the antiscorbutic vitamin," *Z. physiol. Chem.* **204**, 105 (1932); RYGH, O. AND RYGH, A., "Chemical investigation on the antiscorbutic vitamin," *ibid.*, **211**, 275 (1932).

(14) GRANT, A. L., SMITH, S., AND ZILVA, S. S., "Narcotine as the alleged precursor of vitamin C," *Biochem. J.*, **26**, 1628 (1932); HARRIS, J., MILLS, I., AND INNES, J. R., "The chemical identification of vitamin C," *Lancet*, July 30, 1932, pp. 235-7; TILLMANS, J. AND HIRSCH, P., "Über das Vitamin C," *Biochem. Z.*, **250**, 312 (1932); OTT, E. AND PACKENDORFF, K., "Über das vitamin C," *ibid.*, **250**, 312 (1932).

(15) TILLMANS, J., HIRSCH, P., AND JACKISCH, J., "Das Reduktionsvermogen pflanzlicher Lebensmittel und seine Beziehung zum Vitamin C," *Z. Untersuch. Lebensm.*, **63**, 275-83 (1932).

(16) SHERMAN, H. C., LA MER, V. K., AND CAMPBELL, H. L., "The quantitative determination of the antiscorbutic vitamin," *J. Am. Chem. Soc.*, **44**, 165-72 (1922).

(17) SZENT-GYÖRGYI, A., "Observations on the function of peroxidase systems and the chemistry of the adrenal cortex," *Biochem. J.*, **222**, 1387 (1928).

(18) SZENT-GYÖRGYI, A., "On the mechanism of biological oxidation and the function of suprarenal gland," *Science*, **72**, 125 (1930); "On the function of hexuronic acid in the respiration of the cabbage leaf," *J. Biol. Chem.*, **90**, 385 (1930).

(19) SVIRBELY, J. L. AND SZENT-GYÖRGYI, A., "Hexuronic acid as the antiscorbutic factor," *Nature*, **129**, 576 (April 15, 1932); "Hexuronic acid as the antiscorbutic factor," *Biochem. J.*, **26**, 865 (May, 1932).

(20) ZILVA, S. S., "Hexuronic acid as the antiscorbutic factor," *Nature*, **129**, 690 (May 7, 1932).

(21) NELSON, E. K., "The isolation of hexuronic acid," *Science*, **76**, 690 (1932).

(22) COX, E. G., HIRST, E. L., AND REYNOLDS, R. J. W., *Nature*, **130**, 888 (1932); HERBERT, R. W., PERCIVAL, E. G. V., REYNOLDS, R. J. W., SMITH, F., AND HIRST, E. L., "The structure of ascorbic acid," *J. Soc. Chem. Ind.*, **52**, 21-2 (March 10, 1933).

(23) KARRER, P., SALMON, H., MORF, R., AND SCHÖPP, K., "Zur Kenntnis des antiskorbutische Vitamins," *Biochem. Z.*, **258**, 4-15 (Jan., 1933); KARRER, P., SCHWARZENBACH, G., AND SCHÖPP, K., "Über Vitamin C," *Helv. Chem. Acta*, **16**, 302-6 (Mar., 1933).

(24) MICHEEL, F. AND KRAFT, K., "Constitution of vitamin C," *Nature*, **131**, 274 (Feb., 1933); MICHEEL, F. AND KRAFT, K., "Die Konstitution des Vitamin C," *Z. physiol. Chem.*, **215**, 215-24 (May, 1933).

(25) REICHSTEIN, GRUSSNER, AND OPPENAUER, "Die Synthese der *d*-Ascorbinsaure," *Helv. Chim. Acta*, **16**, 561 (May, 1933).

(26) HARRIS, J. AND RAY, S. N., "Specificity of hexuronic acid as antiscorbutic factor," *Biochem. J.*, **26**, 580 (Mar., 1933).

(27) WAUGH, W. A. AND KING, C. G., "The vitamin C activity of hexuronic acid from suprarenal glands," *Science*, **76**, 630 (1932).

(28) SVIRBELY, J. L. AND SZENT-GYÖRGYI, A., "The chemical nature of vitamin C," *Biochem. J.*, **27**, 279-85 (Mar., 1933).

(29) VARGHA, L. V., "Monoacetone hexuronic acid," *Nature*, **130**, 847 (Dec., 1932).

(30) BIRCH, T. W., HARRIS, J., AND RAY, S. N., "A microchemical method for determining the hexuronic acid content of foodstuffs," *Biochem. J.*, **26**, 590 (Mar., 1933).

(31) LOCKWOOD, HARTMAN, AND HARTMAN, "Relation of adrenal cortex to development of scurvy," *Proc. Soc. Exp. Biol. Med.*, **30**, 560 (Jan., 1933).

(32) SZULE, D., "Treatment of Addison's disease with cortigen and lemon juice," *Deut. med. Wochschr.*, **59**, 651 (Apr., 1933).

(33) BIRCH AND DANN, "Estimation and distribution of ascorbic acid and glutathione in animal tissues," *Nature*, **131**, 469 (Apr., 1933).

(34) MARINE AND BAUMANN, "Vitamin C and goiter," *Sci. News Letter* (April 15, 1933).

(35) WAUGH, W. A., BESSEY, O. A., AND KING, C. G., "Preparation of vitamin C from lemon juice," *Proc. Soc. Exp. Biol. Med.*, **30**, 1281-3 (June, 1933).

(36) LEVENE, P. A. AND RAYMOND, A. L., *Science*, **78**, 64 (July 21, 1933).

(37) HANKE, M. T., "Relation of diet to dental caries," *J. Nutrition*, **3**, 433-51 (1931).

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J. PHARMACOL. EXPTL. THERAP. 103: 403-411, 1951.

All CF values to be divided by 2 to correct for inactive isomer in CF standard

J. Pharmacol. Exptl. Therap. 103. 403-11 (1951)

THE EFFECT OF ASCORBIC ACID ON THE URINARY EXCRETION OF CITROVORUM FACTOR DERIVED FROM FOLIC ACID[1, 2]

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Received for publication July 26, 1951

A factor required for the growth of the organism *Leuconostoc citrovorum* (ATCC 8081) was described by Sauberlich and Baumann (1948). Its possible relation to pteroylglutamic (folic) acid (PGA or FA) was recognized by these authors and by Sauberlich (1949), who found that the administration of folic acid increased the urinary excretion of the citrovorum factor (CF) in rats and in man. Conditions which influence the conversion of FA to CF, both *in vivo* and *in vitro* have been under investigation in this laboratory; thus it was shown that aminopterin interferes profoundly with this conversion (Nichol and Welch, 1950b), and that ascorbic acid promotes the formation of CF as indicated by an increase in the amount of CF derived from FA in a liver-slice system (Nichol and Welch, 1950a), and by an increase in the amount of CF excreted in the urine of normal human subjects when ascorbic acid is administered along with test doses of FA (Anker *et al.*, 1950; Welch, 1950).

In the experiments described in this communication the urinary excretion of CF in rats receiving FA was measured to determine the effect of different reducing agents and of aminopterin on this conversion. Also, data are presented which show the influence of ascorbic acid on the urinary excretion of CF in human subjects receiving FA orally in various dosage regimes. Data from these tests in normal human subjects are compared with observations on the excretion of CF by two severely scorbutic men given FA, both without and with ascorbic acid.

METHODS AND RESULTS. *Leuconostoc citrovorum* 8081 was cultured in the medium described by Sauberlich and Baumann (1948). After 18 hours at 37°C. in a forced-draft air-incubator, the amount of growth in 10 ml. assay tubes was measured by turbidimetric readings in a Klett-Summerson photoelectric colorimeter. Synthetic citrovorum factor (leucovorin) in the form of the hydrated barium salt was used as a standard.[3] One unit of CF was defined as the amount required to give half-maximal growth under the conditions described above. In this laboratory, 1.0 microgm. of synthetic CF, expressed as the free acid, is equivalent to approximately 3000 units of CF. "Folic acid" activity was measured by

[1] A preliminary report of some of the material in this paper was presented at the mid-year meeting of the American Society for Pharmacology and Experimental Therapeutics, Boston (Welch *et al.*, 1951).

[2] This investigation was supported, in part, by research grants from the National Institutes of Health, U. S. Public Health Service, and, in part, by the Lederle Laboratories Division, American Cyanamid Company.

[3] Leucovorin, a synthetic form of the citrovorum factor, presumably identical with folinic acid-SF (Shive *et al.*, 1950; Pohland *et al.*, 1951), was supplied by the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of Dr. T. H. Jukes.

titration of the acid produced during the growth of *Lactobacillus casei* (ATCC 7469) for approximately 72 hours in a medium[1] similar to that described by Lepper (1950). Values obtained by the use of this organism include pteroylglutamic acid, citrovorum factor, and possibly derivatives of the latter.

The influence of different reducing agents on the urinary excretion of CF by rats receiving FA is shown in table 1. Adult female rats were placed in metabolism cages equipped with metal collecting-funnels coated with paraffin. A purified cerelose-casein diet (Nichol and Welch, 1950a) containing a basal amount of FA (0.5 microgm. per gm.) was fed for several days before and during the test periods. All doses were administered daily by intraperitoneal injection. At 48-hour intervals the collecting funnels were washed and the urine samples were

TABLE 1

The effect of reducing agents on the urinary excretion of citrovorum factor in rats receiving folic acid

INTRAPERITONEAL INJECTION DAILY		NO. OF RATS	PERIOD OF URINE COLLECTION	URINARY EXCRETION OF CF PER 24 HOURS, MICROGM.	
Folic Acid	Reducing Agent			Average	Range
mgm.	*millimoles/kgm. of body weight*		*Days*		
0	0	12	2	0.21	(0.11–0.43)
1.0	0	12	4	4.08	(2.08–5.17)
1.0	Ascorbic Acid (0.5)	3	4	12.9	(11.8–13.9)
1.0	Glucoascorbic Acid (1.0)	3	4	19.7	(15.3–23.9)
1.0	Cysteine (2.0)	3	4	5.40	(5.05–5.65)
1.0	Glutathione (2.0)	3	4	4.20	(3.40–5.63)
Synthetic CF					
0.25*	0	3	4	11.5	(9.17–15.2)
0.25*	Ascorbic Acid (1.0)	3	4	16.0	(12.0–21.8)

* as the free acid.

stored under toluene in a refrigerator. For each 4-day test period, aliquots of the two collections were combined and the total CF excreted by each rat was determined. The concentration of CF in the urine returned to the average level of that of untreated rats within 24 hours following the test period. Each rat was subjected to two test periods. FA alone was administered during the first 4-day period. Then, following a 48-hour interval without treatment, a reducing agent (freshly neutralized) and FA were injected daily for 4 days. Both ascorbate and glucoascorbate markedly increased the amount of CF excreted in the urine, while cysteine and glutathione, in the large doses used, exerted no significant effect.

When synthetic CF (0.25 mgm.) was administered intraperitoneally, 4 to 6 per cent of the dose appeared in the urine of the rats. When ascorbate was given,

[1] This medium was modified by replacing sodium citrate and K_2HPO_4 with sodium acetate and Salt Solution A.

in addition to CF (table 1), the amount of CF excreted in the urine was increased only slightly; in these small groups of animals, the range of values obtained overlapped with those observed with CF alone.

The effect of intraperitoneal injections of aminopterin (25 microgm. daily) on the urinary excretion of CF in rats receiving FA (2 mgm. daily, I.P.) is shown in figure 1. As in the previous experiment, adult female rats were kept in metabolism cages and the same diet was fed. Urine samples were collected at 48-hour intervals. The excretion of CF during the treatments indicated in figure 1, expressed as the average of the three rats in each group, was followed until each animal had died. The intraperitoneal administration of sodium ascorbate along

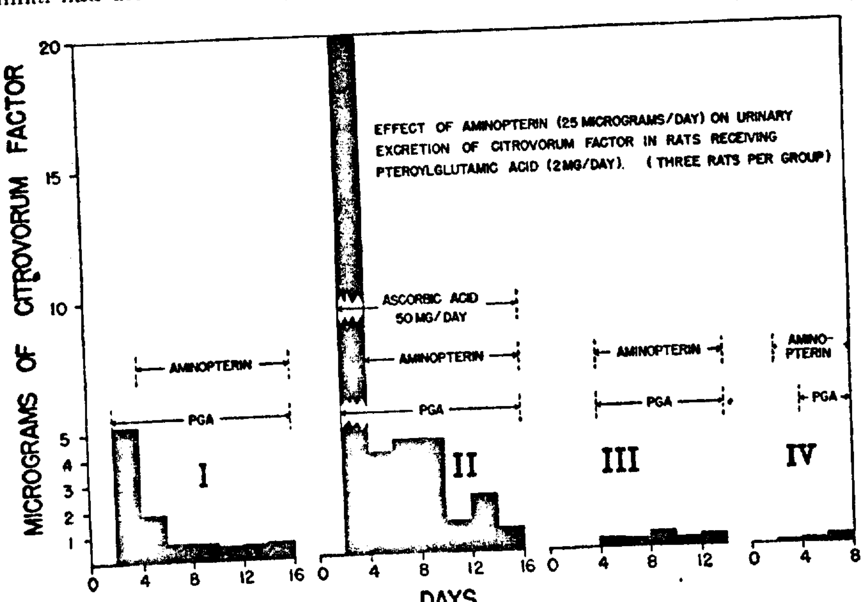

FIG. 1. The effect of aminopterin on the urinary excretion of citrovorum factor in rats receiving pteroylglutamic acid.

with FA (Group II) caused a marked increase in the amount of CF excreted in the urine; this increase was comparable to that observed in the previous experiment. During the treatment with aminopterin, ascorbate (given in addition to FA) increased appreciably the amount of CF excreted (compare Groups I and II). However, even the amount of CF thus formed, of which that excreted in the urine was representative, was insufficient to prolong the life of the animals; as in Group I death occurred within fourteen days following initiation of dosage with aminopterin. The administration of FA simultaneously rather than for two days prior to initiation of dosage with aminopterin (Group III), decreased the survival time by approximately two days, while withholding of FA until two days after the administration of aminopterin was begun (Group IV), led to death of the animals within eight days. All animals died showing the signs typical of aminopterin poisoning (rapid loss of weight, severe diarrhea, porphyrin-caked whiskers). Figure 1 demonstrates that the injection of aminopterin before (IV), at the same time (III), or following the initiation of treatment with FA (I and II), results in an immediate reduction in the amount of CF excreted following uniform dosage with FA.[5]

The amount of CF excreted in the urine of normal human subjects was measured following daily oral doses of FA of 0.5, 5.0 and 50.0 mgm., respectively, during a period of three days. Following this, the effect of simultaneous administration of ascorbic acid along with these amounts of FA was tested in the same individuals (table 2) during a second 3-day period. The actual sequence of events, shown for one dose level in figure 2, was as follows: after a control period of 24 hours without treatment and a 3-day period on FA, no dosage was given for 24 hours. Ascorbic acid (0.75 gm. per day, orally, in three divided doses) was given alone on the sixth day and was continued during the remainder of the experiment. The second 3-day test of FA was begun on the seventh day, while on the final (tenth) day of the experimental period only ascorbic acid was given. Urine was collected in brown bottles during successive 24-hour periods

TABLE 2

The effect of ascorbic acid on the urinary excretion of citrovorum factor in normal human subjects following oral doses of folic acid

DAILY ORAL DOSE		PERIOD OF URINE COLLECTION	AVERAGE URINARY EXCRETION PER DAY	
Folic Acid	Ascorbic Acid*		Folic Acid	Citrovorum Factor
mgm.	*gm.*	*days*	*microgm.*	*microgm.*
0	0	1	4.13	1.07
0.5	0	3	37.4	2.76
0	0	1	5.08	1.22
0	0.75	1	7.17	1.00
0.5	0.75	3	90.6	3.68
0	0.75	1	8.31	1.78
0	0	1		1.46
5.0	0	3		16.0
0	0	1		1.85
0	0.75	1		1.18
5.0	0.75	3		49.7
0	0.75	1		2.67
0	0	1	4.65	1.98
50.0	0	3	9,200.	164.0
0	0	1	232.0	9.15
0	0.75	1	15.1	9.14
50.0	0.75	3	8,400.	450.0
0	0.75	1	250.0	31.5

* 0.25 gm. taken 3 times daily when indicated.

[5] Similar data were presented in a previous publication (Nichol and Welch, 1950b).

and was kept refrigerated under toluene; urine collected during each 3-day test period was pooled prior to analysis. The amount of CF excreted during each period was determined for each individual. On the lowest and highest levels of dosage, eight apparently normal young men served as subjects; on the 5 mgm. dose level of FA, the subjects included four young men and three young women. Although the number of individuals involved was small, there appeared to be no differences in the excretion of CF attributable to sex. In addition to analyses for CF, determinations of FA were made on the urine samples of five individuals given 0.5 mgm. and 50 mgm. doses of FA, respectively.

The effect of ascorbic acid in increasing the amount of CF excreted at each of the three dosage levels of FA is shown in table 2. The increase in CF due to ascorbic acid at the 0.5 mgm.-dose of FA was slight but consistent among the eight individuals and was significant statistically at the 5 per cent level. The three-fold increase in the amount of CF excreted when ascorbic acid was administered together with the 5.0 mgm. and 50 mgm. doses of FA, was highly significant (1 per cent level). In conjunction with the 0.5 mgm. dose of FA, ascorbic acid also caused an increase in the FA-content (activity for *L. casei*) of the urine which was significant at the 5 per cent level; however, with the 50 mgm. dose of FA, simultaneous administration of ascorbic acid caused no significant change in the activity of the urine for *L. casei*.

In view of the definite augmentation of CF-excretion caused by ascorbic acid, when FA is administered to normal men, it appeared desirable to test this conversion in individuals deficient in ascorbic acid. For this purpose it was possible to study two patients with scurvy.

One individual, a 42-year old white male, was admitted to Lakeside Hospital because a leg injury had failed to heal during a 4-week period. At the time of admission the patient showed extreme fatigue, shortness of breath, fever and loss of appetite. Bleeding from the gums and soreness of the tongue were observed and had been noticed by the patient for several weeks. There was subdermal bleeding and discoloration of the right leg which had been injured by a blow at the knee one month previously. Only a trace of ascorbic acid could be detected in the plasma by chemical analysis. The Rumpel-Leede test (7-minute block of venous return in the forearm) was strongly positive. Examination of the blood indicated an increase in clotting time (9 min.) and anemia (hemoglobin 4.6 gm. per 100 ml. and hematocrit 23 per cent). The dietary history revealed that for a period of about three years the patient prepared his own breakfast of toast and coffee, ate one meal each day at noon at a restaurant which served cooked vegetables with the meal but seldom served fresh fruits or salads, and during the afternoon and evening he consumed from 12 to 14 bottles of beer. Clinical improvement was rapid following administration of ascorbic acid (0.25 gm. orally 3 times daily) and on the second day of therapy the Rumpel-Leede test was negative.

The marked difference between the amounts of CF excreted by the scorbutic and the normal subjects is shown in figure 2. Not only was the amount of CF excreted by the scorbutic patient consistently very much less than that found in the urine of normal subjects, but also the simultaneous administration of ascorbic acid caused only very slight augmentation of the elimination of CF, during the three-day period of observation. Daily administration of FA (5 mgm. orally), without ascorbic acid, was continued for three days following the period of therapy with ascorbic acid. A continuation of the gradual increase in the amount of CF excreted per day was observed: 5.6, 8.7 and 8.4 microgm. of CF on the 10th, 11th and 12th days, respectively. It should be noted that this amount is only about one-half that of the average amount of CF excreted by normal individuals given 5 mgm. of FA daily *without ascorbic acid*.

A second patient, a 68-year old white male, had extensive subdermal hemorrhages of the legs and forearms, bleeding of the gums and soreness of the mouth. There was a moderate anemia (hemoglobin 9.2 gm. per 100 ml. and hematocrit 32 per cent) and an increase in clotting time ($8\frac{1}{2}$ min.). Analysis of the plasma indicated only a trace of ascorbic acid. This patient received a daily oral dose

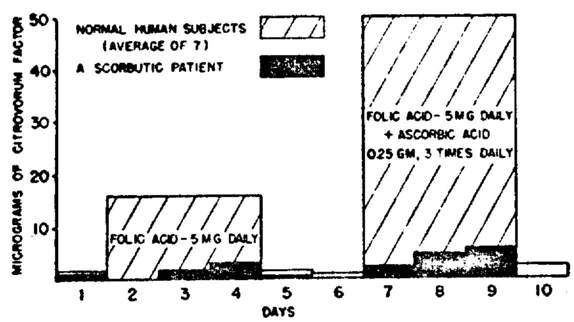

FIG. 2. A comparison of the effect of ascorbic acid on the urinary excretion of citrovorum factor during oral administration of folic acid in normal human subjects and in a severely scorbutic patient.

of FA (5 mgm.) throughout his period of hospitalization. Consecutive 24-hour urine collections were obtained and refrigerated under toluene until assayed for both CF and FA. On the third day, treatment with ascorbic acid (250 mgm. orally 3 times daily) was begun and continued until the patient left the hospital.

No CF could be detected in the urine samples of the first 4 days, that is, on the 1st and 2nd days when FA alone was given and on the 3rd and 4th days when the patient received both FA and ascorbic acid. During the next 4 days, the amount of CF excreted increased (0.7, 7.7, 4.8, 16 microgm. per day) and by the 6th day of treatment with ascorbic acid, the excretion of CF had reached a fairly constant level—15, 17, 16, 13, and 15 microgm. per day on the 7th, 8th, 9th, 10th and 13th days, respectively, of treatment with both FA and ascorbic acid.

The folic acid activity of the same samples of urine showed that on the two days when FA alone was administered, 9 and 14 per cent, respectively, of the test dose was excreted. On the next two days during treatment with ascorbic acid and FA, 42 and 33 per cent, respectively, of the doses were excreted. No CF activity could be detected in the urine on any of these four days. On three consecutive days when the CF excretion had reached a constant level (16, 15 and 17 microgm. per day) the amount of folic acid in the urine was 48, 46 and 42 per cent of the test dose, on the respective days.

DISCUSSION. The data presented in figure 1 offer a logical explanation for the well-known partial protection against the toxicity of aminopterin which is afforded by preliminary treatment with FA. This figure shows that the synthesis of CF from FA, as reflected by urinary elimination, is promptly blocked by the FA-analogue. Only when FA was administered prior to the antagonist, either without or with ascorbic acid, was the lethal action of aminopterin delayed and a significant excretion of CF noted.

In a previous paper (Nichol and Welch, 1950b) it was shown that aminopterin blocks almost completely the conversion of FA to CF by liver slices and that animals given otherwise lethal doses of aminopterin could be protected completely by CF under conditions where relatively massive doses of FA were impotent. In that paper it was pointed out that aminopterin not only prevents the metabolic alteration of FA but also competes with the product (CF) derived from FA. Recently, Burchenal and Babcock (1951) have stressed the role which amethopterin plays as an antagonist of CF, an effect previously noted by Brockman *et al.* (1950) in mice receiving aminopterin. However, it must be emphasized that with either of these folic acid analogues the efficiency of the interference with the formation of CF from FA is of a very much higher order of magnitude than is the interference with the utilization of the factor, although the lethal effects of these analogues ultimately must be attributed to their *competitive* inhibition of the utilization of CF.

The conversion of FA to CF by liver slices *in vitro* is augmented markedly by the addition of either ascorbate or its analogue, glucoascorbate (Nichol and Welch, 1950a). The latter compound is a powerful reducing agent, but it possesses little antiscorbutic activity; on the other hand, such potent reducing agents as cysteine and glutathione have little or no influence on the conversion *in vitro*. The data obtained *in vivo* in rats show that glucoascorbic acid, like ascorbic acid, is able to augment the excretion of CF when it is administered together with FA. In view of the findings *in vitro*, these results may be interpreted as indicating that ascorbic acid and its analogue facilitate the conversion of FA to CF. Were the data from the experiments *in vitro* not available, the effect of these relatively strong acids in increasing the urinary excretion possibly could have been attributed to an effect on renal tubular mechanisms. The possibility of the participation of such a mechanism cannot be entirely excluded since the urinary excretion of FA following very small doses appears to have been increased significantly by the administration of large doses of ascorbic acid (table 2). However, with large doses of FA (50 mgm. daily) ascorbic acid caused no augmentation of urinary elimination of FA, although it did result in a three-fold increase in an already marked urinary elimination of CF. That the possible renal factor is of little importance in explaining the results with CF also is indicated by the lack of a significant effect of ascorbic acid on the urinary excretion of CF, *following dosage with CF*.

It was suggested that, in the enzymatic conversion of FA to CF (Nichol and

Welch, 1950a, b), ascorbic acid is concerned with a reductive step. The likelihood of such a role for ascorbate is supported by the reports of catalytic hydrogenation of FA as a step in the synthesis of CF (Shive *et al.*, 1950; Brockman *et al.*, 1950; Pohland *et al.*, 1951).

Numerous clinical and experimental observations have indicated that ascorbic acid plays a role in certain anemias (Vilter, 1947). Certain instances of a clinical response of pernicious anemia to therapy with ascorbic acid have been reported (Dyke, 1942) and May and coworkers (1950) have demonstrated that a deficiency of ascorbic acid favors the development of megaloblastic anemia in monkeys and human infants. An anemic condition has frequently been noted in scorbutic individuals (Vilter, 1947). The very low excretion of CF in the scorbutic patient appears to reflect an abnormality in the metabolism of folic acid in the tissues. Adult patients with scurvy are but rarely seen, and these observations on only two such cases cannot be interpreted too broadly. However, Burchenal and Schilling (1950) also have studied the urinary excretion of CF by a scorbutic individual given FA (10 mgm. daily), without and with ascorbic acid. The sequence of events was somewhat similar; an immeasurably low output of CF was gradually increased when daily doses of ascorbic acid totaling one gram were given during a period of one week. Despite this regime, the values for CF, during the period of observation, did not reach those characteristic of the normal individual. This may represent the marked storage of CF which occurs, or the prolonged lack of ascorbic acid may have produced a change in the tissues which is not corrected immediately by the administration of massive doses of ascorbic acid.

The need for ascorbic acid is not alleviated by the administration of folic acid (Silverman and Mackler, 1951). Although the megaloblastic anemia of scorbutic monkeys can be alleviated by folic acid, or by very much smaller doses of CF (May, Sundberg and Schaar, 1950), other important manifestations of scurvy are not relieved. However, correction of the scurvy with ascorbic acid abolishes megaloblastic anemia without the administration of additional FA or CF (May *et al.*, 1950). These findings give further emphasis to a role of ascorbic acid in the metabolic activation of folic acid.

It should be pointed out that the absence of CF from the urine of a scorbutic patient during the first four days of treatment with FA was not due to an inability to absorb FA, since on these days 9 to 42 per cent of the test dose was excreted in the urine as FA. The failure of scorbutic patients promptly to excrete larger amounts of CF, following dosage with ascorbic acid in addition to FA, could reflect either an abnormality in the conversion of FA to CF which only gradually is corrected by vitamin C, or a marked removal of CF from the blood by deficient tissues. That the latter may be the more likely explanation is suggested by the results in scorbutic monkeys (May, Sundberg and Schaar, 1950).

SUMMARY

The urinary excretion, by rats and human subjects, of a biologically active metabolic alteration product (citrovorum factor) of pteroylglutamic (folic) acid

has been studied. The relationship between size of dose and magnitude of excretion is described. The excretion of the citrovorum factor was augmented by the administration of ascorbic acid, an effect attributed to a participation of ascorbic acid in the enzymatic conversion of folic acid to citrovorum factor (a reduced form of the vitamin). In rats, glucoascorbic acid was found to be as active as ascorbic acid, while cysteine and glutathione were without significant activity. Aminopterin inhibited markedly the urinary excretion of citrovorum factor following the administration of folic acid to rats. The excretion data account for the effect of previous dosage with folic acid in diminishing the toxicity of the analogue; such prior treatment with the vitamin, by saturating the tissues with the citrovorum factor, increases the period of survival of the animals. Emphasis is placed on the fact that the 4-amino analogues of folic acid more efficiently interfere with the formation of citrovorum factor than with its utilization.

Two adult patients with scurvy excreted only small amounts of citrovorum factor following the administration of folic acid, and this excretion was but little augmented by therapy with large doses of ascorbic acid, in contrast to normal human subjects.

Acknowledgment: The authors are indebted to Dr. Max Miller and members of the resident house staff of the Department of Medicine, Lakeside Hospital, whose interest and cooperation made possible the study of the scorbutic patients. We acknowledge gratefully the technical assistance of Sarah L. Rhodes, Caroline A. McKalen and Marilyn W. Slote.

REFERENCES

ANKER, R. M., BOEHNE, J. W., AND WELCH, A. D.: Fed. Proc., **9:** 351, 1950.

BROCKMAN, J. A., JR., ROTH, B., BROQUIST, H. P., HULTQUIST, M. E., SMITH, J. M., JR., FAHRENBACH, M. J., COSULICH, D. B., PARKER, R. P., STOKSTAD, E. L. R., AND JUKES, T. H.: J. Am. Chem. Soc., **72:** 4325, 1950.

BURCHENAL, J. H., AND BABCOCK, G. M.: Proc. Soc. Exper. Biol. and Med., **76:** 382, 1951.

BURCHENAL, J. H., AND SCHILLING, R. F.: Personal communication, 1950.

DYKE, S. C., DELLA VIDA, B. L., AND DELIKAT, E.: Lancet, **2:** 278, 1942.

LEPPER, H. A., Editor: Official Methods of Analysis of the Association of Official Agricultural Chemists, Washington, D. C., Seventh Edition, 784, 1950.

MAY, C. D., NELSON, E. N., LOWE, C. U., AND SALMON, R. J.: Am. J. Dis. Child., **80:** 191, 1950.

MAY, C. D., SUNDBERG, R. D., AND SCHAAR, F.: Proc. Cent. Soc. Clin. Res., **23:** 71, 1950.

NICHOL, C. A., AND WELCH, A. D.: Proc. Soc. Exper. Biol. and Med., **74:** 52, 1950a.

NICHOL, C. A., AND WELCH, A. D.: Proc. Soc. Exper. Biol. and Med., **74:** 403, 1950b.

POHLAND, A., FLYNN, E. H., JONES, R. G., AND SHIVE, W.: Abstracts of Papers, Am. Chem. Soc., 18M, 1951.

SAUBERLICH, H. E.: Arch. Biochem., **24:** 224, 1949.

SAUBERLICH, H. E., AND BAUMANN, C. A.: J. Biol. Chem., **176:** 165, 1948.

SHIVE, W., BARDOS, T. J., AND ROGERS, L. L.: J. Am. Chem. Soc., **72:** 2817, 1950.

SILVERMAN, F. N., AND MACKLER, B.: Proc. Soc. Exper. Biol. and Med., **76:** 574, 1951.

VILTER, R. W.: in LEJWA, A., Symposium on Nutrition of the Robert Gould Research Foundation, Cincinnati, Ohio, **1:** 179, 1947.

WELCH, A. D.: Trans. Assoc. Am. Physicians, **63:** 147, 1950.

WELCH, A. D., NICHOL, C. A., ANKER, R., AND BOEHNE, W.: THIS JOURNAL, **101:** 37, 1951.

White, A., P. Handler and E.L. Smith. 1968
Principles of Biochemistry, 4th Edition
McGraw-Hill Book Company, New York, pp. 1044-1047

Anal. Chem. 44(1267-1269), 1972.

Continuous Determination of Ascorbic Acid by Photobleaching of Methylene Blue

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THE PHOTOLYSIS of a flowing stream to produce a reactive titrant has been reported (*1*). It was found that a solution could be completely or partially photolyzed with precision of 1% or better (See Table II, Reference *1*). However it was then necessary to add the photolyzed solution to a batch sample; this is cumbersome and time consuming for routine analysis. Because the photolysis step proved to be precise to 1%, one could propose a method in which the sample is added to the stream prior to photolysis and the analytical measurement then made on the combined stream of sample and photoreagent after the photochemical reaction.

Since photons can be added to a flowing stream without physically breaking into the line, photolysis should be a convenient sample treatment method for use in continuous analytical methods. The automation of analysis using flowing streams has been reviewed by Blaedel and Laessig (*2*); the definitions given in their review will be followed here. The batchwise determination of ascorbic acid (hereafter AA) *via* the photobleaching of methylene blue (hereafter MB) has been performed photometrically (*3*) and titrimetrically (*4*) and was chosen as the test model for continuous photochemical analysis. An advantage of this particular photochemical system is that the leuco form of MB, the product formed in the analytical reaction, can be returned to the intensely colored form by contact with atmospheric oxygen (*5*). This means that a given batch of MB can be used more than once as the carrier stream for AA determinations. On the other hand, oxygen dissolved in the carrier stream of MB can partially offset any photobleaching due to AA, and it may be necessary to remove O_2 from the system in some cases.

In a preliminary check of the system, we found that ultraviolet radiation from a mercury arc did not bleach the AA–MB system; the photoactivity is found in the 655 nm absorption band of MB (*5*). The reaction is so light sensitive that a valid absorption spectrum of AA–MB mixtures cannot be obtained because of photobleaching during the time of the wavelength scan.

EXPERIMENTAL

Apparatus. A diagram of the flow system assembled for this study is shown in Figure 1, and is very similar to the previously described device used for external photochemical titrant generation (*1*). Only changes from previous apparatus and procedures will be described here. R_1', P, and M provide a means to introduce a sample of AA (1-ml volume) into the carrier stream of MB. After sample injection, the stopcock position at point M is reversed and the sample is swept through the system by MB solution supplied by gravity feed from R_1. The photolysis is accomplished with a 750-W projector bulb (Sylvania Electric, Model-DEC) positioned down the center axis of a quartz spiral tube (6-mm i.d., 25-ml capacity). The extent of photolysis is independent of intensity when the lamp is operated at any applied power between 600 and 750 W. The lamp and spiral tube are surrounded by an aluminum housing (RZ) to increase the intensity *via* reflections (*1*). A high-volume rotary blower, B, is used to force air over the lamp and spiral tube. Solutions emerge from RZ significantly warmer than room temperature, but this was not a problem. The extent of photobleaching of the MB is measured spectrophotometrically with S (Bausch & Lomb Spectronic 20, 655 nm) and the increase in %*T* is recorded (100 ppm solution of MB = 0.7%*T*). The flow-through cell (8-mm i.d.) is of all-glass construction and is similar to a widely used design (*6*). The total flow rate is measured with F_2 (Fischer & Porter, No. 01N-150-A) and is adjusted *via* needle valve V (*1*). F_1 (Manostat No. 36-541) serves to assure that there is a forward flow of a few ml/min from R_1' even when most of the carrier flow is supplied from R_1. When a sample is introduced at stopcock M, F_1 is pegged full-scale; the flow rate during sample injection is read using F_2. R_2 is used to catch photolyzed solution; the contents of R_2 can be pumped back to R_1 (this connection is not shown in Figure 1) after the MB color has fully returned (*ca.* 2 hours).

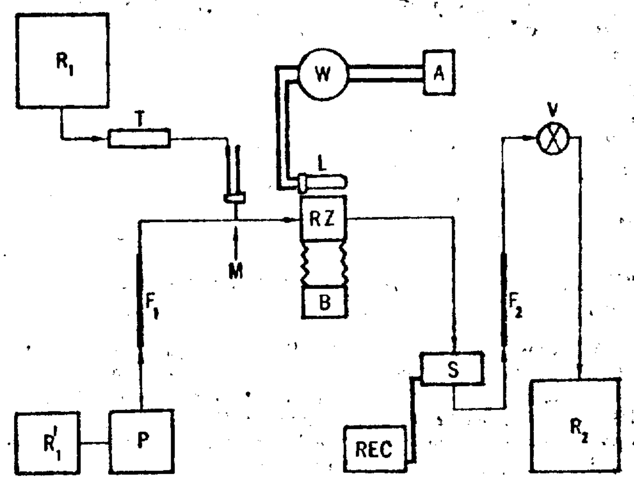

Figure 1. Diagram of apparatus for continuous analysis *via* photobleaching

R_1, Reservoir (20 l.); R_1', Reservoir (2 l., connected to R_1); T, Particle trap (fritted glass); P, pump; F_1, F_2, Flow meters, (0–15 and 0–150 ml/min, respectively); M, Double oblique stopcock and mixing "T"; A, Autotransformer; W, Wattmeter; L, Photolysis lamp; RZ, Reaction zone (spiral tube surrounded with aluminum reflector); B, Air blower; S, Spectrophotometer; REC, Recorder; V, Needle valve; R_2, Reservoir (20 l., for reuse of MB solution)

(1) H. D. Drew and J. M. Fitzgerald, ANAL. CHEM., **41**, 974 (1969).
(2) W. J. Blaedel and R. H. Laessig in "Advances in Analytical Chemistry and Instrumentation," Vol. 5, C. N. Reilley and F. W. McLafferty, Ed., John Wiley and Sons, New York, N.Y., 1966, pp 69–168.
(3) H. Lund and H. Lieck, *Klin. Wochenschr.*, **16**, 555 (1937).
(4) H. Lund and E. Trier, *ibid.*, **18**, 80 (1939).
(5) J. D. Margerun, A. M. Lackner, M. J. Little, and C. T. Petrusis, *J. Phys. Chem.*, **75**, 3066 (1971).
(6) J. S. Fritz and G. S. Schenk, "Quantitative Analytical Chemistry," 2nd ed., Allyn and Bacon, Inc., Boston, Mass., 1969, p 578.

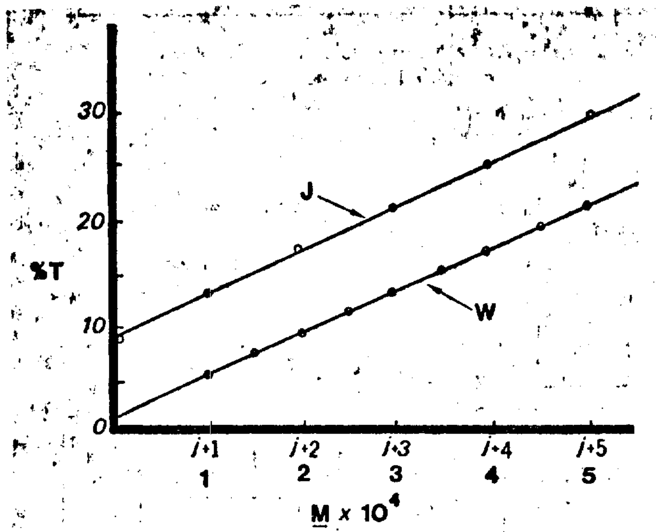

Figure 2. Calibration plots for ascorbic acid determination in orange juice

%T, Transmittance of 25 ppm MB carrier solution when photobleached with AA solution
J, Standard addition line; use upper concentration axis. AA from 3 ml of juice diluted to 25 ml represented by "j"
W, AA standards in water; use lower concentration axis. Data points are averages of five or more injections

Reagents. Reagent grade chemicals and distilled-deionized water were used throughout. The carrier solution of MB is adjusted to pH 2 (*4*) with HCl. It is significant to note that one 20-l. batch of solution may be used repeatedly. Buildup of AA photooxidation products, the presence of reasonable amount of diverse substances used in the interference tests, and dilution all resulted in no significant change in the photobleaching when the MB was treated with a fresh AA sample. One batch of 100 ppm MB solution was used for over three thousand individual determinations before it became too contaminated.

Commercial vacuum cans of unsweetened orange juice and 500- or 250-mg (USP) vitamin C tablets were used. Standard AA solutions were prepared from solid reagent daily. Serial dilutions were then made to establish the slope of a working line. Interference tests were run by adding solid reagent to standard AA solutions followed by sample injection and photobleaching measurement in the usual manner.

Procedure. Prior to running a set of samples, the flow rate must be adjusted to a compromise value which reflects the requirements of two opposing sets of facts. The time which the sample spends in the photolysis zone and dilution of sample with MB carrier are favored by a slow flow rate. But a sharp photometric recorder trace and the time interval between samples are favored by fast flow rate. The interaction of these time-dependent variables is complex and has been discussed previously (*1, 2*). It should be noted here that conservation of carrier solution is not a consideration in selection of flow rate because the MB is recycled. In conventional continuous analysis, the solution is discarded following the measurement step; slow flow rates obviously favor economy and convenience.

The flow rate provided by the pump from R_1' is adjusted to about 50 ml/min while sample is fed into the stream by gravity (1 ml injected in 20 sec). Faster flow rates slow the sample feed because of higher back pressure and result in excessive dilution. Fifty ml/min provides a 1:17 (AA to MB) dilution. When the sample injection stopcock position is reversed, almost all the MB flow comes from R_1; the contribution from R_1' drops to only 1–2 ml/min. The total flow rate is adjusted to about 80 ml/min with valve V. Since the sample is combined with MB solution from R_1', only this small reservoir must be flushed with nitrogen if an oxygen-free determination is desired. The larger volume of R_1 serves only as carrier between samples and need not be deaerated.

Once the flow rates for injecting the sample and carrying the diluted sample are set, sample solution is used to fill the standpipe of stopcock M to a fixed volume and then allowed to drain into the mixing "T" at a reproducible rate. About 1 min later, the maximum recorder deflection is obtained and another sample may then be injected; thus samples can be run nearly as fast as the standpipe can be filled.

The maximum increase in $\%T$, read from the recorder trace, is plotted *vs.* concentration of AA in the original sample and linear calibrations are obtained (Figure 2). The concentration range following the above procedure with 100 ppm MB carrier is 4 to 50m*M* AA. Concentration between 0.3 and 1.3m*M* can be determined if 25 ppm MB carrier is used. Least-squares fits of data are useful for statistical analysis of the results (*1*), but calibration plots suffice for routine measurements.

The limit of detection for AA can be improved by use of even more dilute MB carrier solution. For example, using 5 ppm MB, $5 \times 10^{-4}M$ AA could just be distinguished from the increase in $\%T$ caused by the injection of a water blank. If 100 or 25 ppm MB is used, a blank causes no observable change in $\%T$. The upper AA concentration limit using 5 ppm MB carrier solution is less than 10 times the detection limit because of nonlinearity in photobleaching kinetics at large $\%T$ values (*1*). Thus, continuous analysis is possible at lower AA concentrations at the cost of a decrease in the concentration range covered.

Measurements of AA in orange juice requires dilution with water and centrifugation to remove pulp. Prior to developing the routine procedure, we determined the range of volume per cent of juice for which linear photobleaching occurred. This was accomplished by serial dilution of juice, centrifugation, and injection of 1-ml aliquots into 25 ppm MB solution. We found that cans from several different lots of juice all gave linear working curves of $\%T$ *vs.* volume of juice between 8 and 25%. (Least-squares treatment gave relative standard errors of slopes of less than 2%.) Next, fixed amounts of juice were combined with varying amounts of standard AA, centrifuged, and photobleaching of aliquots was measured. The slope of the standard addition working line was the same as that for AA in water alone (Figure 2). The serial dilution and standard addition experiments show that the bleaching of MB by AA in juice is identical to that of AA in water, provided one makes a suitable dilution of the viscous juice and removes the pulp prior to sample injection. For routine analysis, aliquots of 10 to 15 volume per cent juice can be used with 25 ppm MB and the AA concentration read directly from a working line established with AA in water (Figure 2).

Tablets containing either 500 or 250 mg of vitamin C were analyzed by dissolution in a few ml of 6*M* HCl followed by appropriate dilution for injection into 25 ppm MB carrier. This was accomplished by dilution of tablets to 250 ml; then a 5:100 or 10:100 dilution was made for the 500- and 250-mg tablets, respectively. The second dilution also served to dilute out turbidity due to undissolved binder from tablets. Dilutions of a standard 1 mg/ml AA solution were used for the working line similar to Figure 2.

RESULTS AND DISCUSSIONS

The effect of dissolved oxygen was studied by saturating reservoir R_1' with either nitrogen or oxygen. Using the bleaching of air-saturated 100 ppm MB by 10m*M* AA as a reference point, a 21% relative gain in amount of bleaching for the same amount of AA resulted when N_2 was used and a 19% relative loss in bleaching resulted from O_2 saturation. Therefore for maximum sensitivity, the sample should be mixed with oxygen-free MB. However, for routine measurements, air-saturated MB was satisfactory; least-squares reduction of data, summarized in Table I, demonstrate the day-to-day reproducibility. Our laboratory is both temperature and humidity controlled, so the oxygen concentration in the MB reservoirs is essentially constant from day to day. Air-saturated MB was used for all AA determinations reported here. A typical working line is shown in Figure 2. Least-squares reduction of other data are given in Table I.

The kinetics of photolysis of a solution passing through a tubular reactor has been discussed elsewhere (*1*, *7*); for continuous analysis it is only necessary that the kinetics be reproducible (*2*), and data summarized in Table I demonstrate that this was found to be so, even on different days. If the recorder is adjusted to read 0 to 10%*T* full scale, the last column of Table I (*8*) then shows that the concentration error in a determination should be around ±3%, which is reasonable for a rapid continuous photometric analysis.

Table II presents a summary of the interference studies. No substance interfered by competitive bleaching of the MB, although tryptophane and tryosine are reported to do so at pH 10 (*9*). Note that all the aromatic compounds studied (tryptophane, tyrosine, and benzoic acid) interfered seriously by prevent MB bleaching, but only one sugar, mannose, was a serious interference. For analysis of real substances, conclusions are: AA in orange juice (without preservatives such as benzoate) should not be affected by the other substances, mainly sucrose and glucose, present. Artificial juices enriched with AA and stabilized with benzoate would be more difficult. Artificial juices usually contain about $10^{-3}M$ AA while orange juice is more concentrated, which favors the orange juice analysis with dilution such as used in the recommended procedure. Urine analysis is not possible without pretreatment because of the interference of urea and traces of aromatic compounds.

Measurements of AA in orange juice are facilitated by dilution and the sensitivity of MB bleaching allows one to do so. Removal of pulp solids by centrifugation improved precision of %*T* readings and prevented fouling of the flowmeter and needle valve. Therefore dilution could be used both to facilitate removal of pulp and also for standard addition experiments. A linear increase in %*T* due to photobleaching was found over the range 8 to 25 volume per cent of orange juice in water. With the recommended procedure, it was possible to determine AA in juice using a working curve prepared with AA standards in water. Verification of the concentration was carried out with standard addition. The two lines shown in Figure 2 are parallel; least-squares slopes and standard errors are 0.423 ± 0.004 (60 observations) for AA in water and 0.417 ± 0.008 (20 observations) for the standard addition experiments. Several lots of canned juice were analyzed and AA concentrations between 2.3 to 2.8m*M* were found; a recently reported spectrophotometric method yielded similar results (*10*).

Table I. Day-to-Day Reproducibility of Calibration Plot for Ascorbic Acid Determination

Day	No. of detns	Correl. coeff.	Slope[a]	Std error[a] of slope	Std error[b] of estimate
1	32	0.9975	1.921	0.025	0.324
2	24	0.9976	1.927	0.028	0.320
3	45	0.9975	1.922	0.020	0.348

[a] From least-squares data treatment. Units of $\Delta\%T/\mathrm{m}M$ AA.
[b] Root-mean-square deviation of sample points from least-squares line. Units of $\Delta\%T$. See reference *8* for details.

Table II. Interference Levels for Continuous Photochemical Analysis of Ascorbic Acid

Substance added	Interference level[a]
Aspartic acid	1:50
Benzoic acid	1:1
Citric acid	1:10
Cysteine	1:8
Fructose	1:10
Glucose	1:100
Glutamic acid	1:10[b]
Lactic acid	1:10
Mannose	1:1
Succinic acid	1:100[b]
Sucrose	1:10
Tartaric acid	1:70
Tryptophane	1:1
Tyrosine	1:1
Urea[c]	1:3

[a] Mole ratio of AA to interference which results in less than 5% relative decrease in amount of photobleaching of 100 ppm MB when 8m*M* AA is injected.
[b] No interference at solubility limit of diverse substance.
[c] Solution adjusted to pH 2.

Results for measurements of commercial vitamin C tablets were quite good. A total of seven 500-mg tablets analyzed on two different days yielded an average value of 497 mg with an overall standard deviation of 2.3 mg. Three 250-mg tablets gave an average value of 247 mg with standard deviation of 1.2 mg. Standard deviations for individual tablets were less than 1 mg in all cases.

The fact that the MB photolysis can be reversed by air oxidation enables one to recycle the carrier solution many times. Thus an AA working curve prepared for a given 20-l. batch of MB can be used for many subsequent measurements. The fact that the solution is not discarded allows one more freedom in selecting flow rates which optimize photolysis and photometry. The convenience and precision of photolytic treatment for use in continuous analysis is worthy of further investigation with other photochemical systems.

(7) A. E. Cassano, *Rev. Fac. Ing. Quim., Univ. Nac. Litoral*, **37**, 469 (1968).
(8) W. J. Dixon and F. J. Massey, Jr., "Introduction to Statistical Analysis," 2nd ed., McGraw-Hill, New York, N.Y., pp 191–3.
(9) L. Weil, *Arch. Biochem. Biophys.*, **110**, 57 (1965).
(10) B. Jaselskis and J. Nelapaty, ANAL. CHEM., **44**, 379 (1972).

RECEIVED for review April 14, 1971. Resubmitted October 18, 1971. Accepted February 22, 1972. Financial support for this work was provided by Grant E-384 from the Robert A. Welch Foundation, and Faculty Research Grant RIG-7003 from the University of Houston.

Reprinted from the BRITISH MEDICAL JOURNAL
10 *June* 1967, 2, 698-699

Ascorbic Acid and Colds

SIR,—I read with considerable interest the article by Dr. Georgina H. Walker and her colleagues (11 March, p. 603), in which they described their studies on the use of ascorbic acid for the treatment of symptoms in the common cold. While they are to be congratulated on a critical examination of this difficult problem, I feel that it is important to question the interpretation of some of their results. Their studies were conducted on a selected group of volunteers, but they did not indicate their sexes or ages. They used an artificial method for infection of their subjects consisting of the intranasal instillation of a saline suspension virus which they claim produces comparable symptoms to those experienced by people exposed to natural infection. Their numbers were small, since only 36 patients developed colds out of the 91 subjects who were inoculated, yet no information was provided about whether the subjects had all received a comparable intake of ascorbic acid prior to the investigation. For three days preceding intranasal instillation of three selected viruses which frequently cause colds, the experimental subjects received 3 g. of ascorbic acid, but the experimental results provided no evidence that ascorbic acid affected the incidence or severity of colds, or the duration or type of symptoms in the subjects under these conditions.

In the general population colds develop in a rather more random fashion, and a variety of factors may influence their incidence and course. Among these factors the consumption of large doses of ascorbic acid in the very early stages of a cold is popularly believed to have a therapeutic effect in suppressing symptoms · Dr. Walker and her colleagues used this type of medication in their studies. The effect of ascorbic acid has been investigated in Dublin in large field surveys during two winter periods of six months each since 1965. In these surveys tablets have been given to the subjects daily, and the ability of ascorbic acid to prevent the occurrence of colds in a population—that is, its prophylactic effect—has been studied. The results of these investigations are not yet analysed completely. Nevertheless, the initial results in these prophylactic trials do not appear to support the totally negative view which arises from the experimental work on the therapeutic effect of ascorbic acid which has been reported from Salisbury. These results will be described when the analysis has been completed.—I am, etc.,

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Printed in Great Britain by J. Smethurst & Co. Ltd.,
35 Rothschild Road, London, W.4.

Applications of Drug Evaluation in Ireland

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SIR John McMichael has given us a fascinating account of the problems inherent in drug evaluation from the point where the scientist first comes across a new discovery to the final stage of treatment evaluation in the population on a large scale. In a country like Ireland which is now considering the best methods for introduction of drug control, evaluation of drugs must play a large part in our thinking. Such evaluation is generally carried out by professional scientists, the chemical pharmacologist, the general pharmacologist, and the human pharmacologist who examines the pharmacodynamics of the drug in human beings. The clinical pharmacologist carries out more extensive trials in samples of the population, and finally the drug is released for therapeutic use by the clinician who prescribes the drug to his patients. The prescription of a drug by a doctor, or the advice and encouragement about drug-taking by an unqualified individual, the administration of the drug, and its consequent action in the person who received it, are the outcome of the knowledge and experience gained from earlier evaluation of the drug.

The results of its administration may be far-reaching. Effective therapy for the disease may be achieved and the patient returns satisfied to his place in society. The therapy may not be successful because the wrong drug is used, the dose was not correct or the side-effects were more serious than anticipated. The consequences become obvious in the unsatisfied patient and his disgruntled social circle. If the effects produced by the administration of the drug have sufficient repercussions in the community the ripples set in motion may spread far. This is illustrated in Figure 1 and is exemplified by the history of the thalidomide incident. In our society self-administration of many drugs is permissible and fashionable; cough mixtures, purgatives, aspirins and other mild analgesics are taken frequently and by many people. Such drugs remove undesired symptoms and enable an individual to resume his place in society, but often their consumption apart from medical need or in unnecessary quantities, that is their abuse, leads to a greater or lesser degree of drug dependence (WHO 1965). It is fashionable, but not permissible to take other drugs in an uncontrolled fashion in Western culture, such as amphetamines, cannabis and heroin. This fashion started in serious proportions about ten years ago in the United Kingdom, and now is characteristic of a section of the adolescent population in these islands. These drugs are taken to produce various desired pharmacological actions which the individual and his culture believe will endow attainable supernormal attributes of a temporary nature.

Evaluation of Drugs

The evaluation of drugs is of interest to the pharmacologist from the point where its metabolism is first investigated in isolated tissues, until the stage when the drug is used, misused, or abused by the community at large. In discussing the subject it is relevant to consider it in relation to experience in Ireland. The population of Ireland is a relatively small compact stable community. It should be an ideal community on which to carry out long-term studies of the side-effects of drugs and so examples will be chosen in relation to the evaluation of drugs which can be applied in the field of social pharmacology. This can be defined as the theory and practice of pharmacology in which the available social resources in relation to drug development, use and control, are mobilized in order to maintain and modify the health of the individual in society and the health of society as a whole.

Sir John referred to the problems of establishing the value of treatment. This problem is complicated and difficult to solve particularly in the case of drugs which act on the central nervous system. It is maintained that the modern methodology of the double-blind clinical trial enables the clinical pharmacologist to solve this problem. Investigations which we have carried out demonstrate the difficulties of assessment of drugs acting centrally. We treated a group of individuals in an institution who complained of sleeplessness. They each received a barbiturate and a dummy tablet. We repeated the investigation three times in succession on the same

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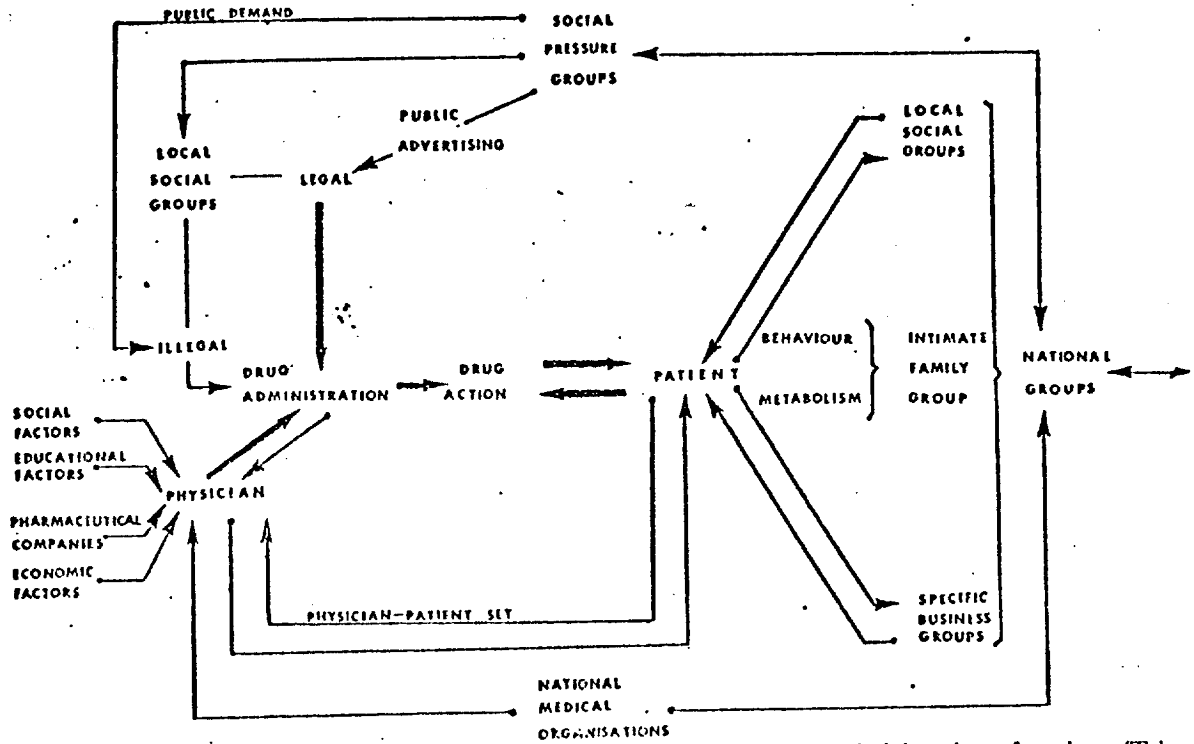

Figure 1. The repercussions set in motion in the community following the administration of a drug (Taken from Wilson, 1967*b*).

individuals. When we examined the results of the effects of the tablets, to our amazement, we found that we could grade the patients by the number of occasions on which they acted as placebo-reactors. The placebo reaction is not a constant effect on the same individual on successive occasions. Objective methods of evaluating treatment are difficult to assess on individual patients under constant conditions. How much more difficult is assessment on populations?

There has been some discussion in this symposium about the effects of drug advertising in the medical and lay press. In the work in Liverpool, to which Professor Andrew Wilson and Dr Cahal referred, we carried out a series of investigations in order to find out what factors affect the prescribing habits of doctors. It is generally assumed that the majority of drugs, taken by individuals in our society, originate from doctors' prescriptions. However, the evidence on which this assumption is based is by no means conclusive. Aspirin is used by about 88 per cent of the male population and 99 per cent of the female population under the age of 30. Perhaps the most interesting aspect of the use of this drug is that 48 per cent of the symptoms for which adolescents in the age-range 15–20 years administer aspirins to themselves, are unaffected or made worse by the treatment. It is clear that any beneficial action which the recipients claim is produced under these circumstances must be due to a placebo effect (Wilson, 1967*a*). Aspirin may cause toxic effects in such individuals, but phenacetin which is used in a similar way, can produce more serious and often fatal effects. In the case of both these drugs, self-administration results in wide-spread abuse, and is to a large extent based on encouragement of their use by public advertisement. This can only be condemned because the symptoms for which aspirin is used often have no pharmacological justification.

Sir John has described the importance of relating the results of animal experiments to the effects produced when a drug is first administered to human beings. Investigations of a drug's action in animals is not always capable of providing the answers about the way in which it works in human beings when specific actions are being investigated. There have been reports that the trichomonicidal agent, metronidazole, has the property of reducing the desire to drink alcohol. The proof for such an action is difficult to obtain and the way in which a selective effect on the consumption of a specific article of diet could be produced is difficult to explain. There is no evidence that a biochemical mechanism like that of disulfiram is involved. Experiments in rats showed that what Richter described as the taste threshold might influence their consumption of alcohol (Rick and Wilson, 1966). This observation provided a clue about how metronidazole might work, but only by testing the drug in man could the evidence be obtained about a subjective effect of this nature. Experiments subsequently carried out in this department have demonstrated that

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the action of metronidazole in reducing the desire for alcohol in human beings is, in fact, related to their taste for alcohol. Objective tests of alcohol concentrations in the blood give supporting evidence for the conclusions which can be drawn about the effects of the drug on the estimates by the subjects about their taste threshold.

Sir John pointed out with reference to tranylcypromine that adverse reactions can arise to the disadvantage of the patient when a drug reacts with cheese which is a normal article of diet. It is of interest however to realize that the opposite effect can be produced by the appropriate use and selection of drugs in the population. Articles of diet which may have an undesired or deleterious effect on an individual, such as alcohol, can be rendered unpleasant or undesirable by the use of drugs like disulfiram or metronidazole. The possibility of using drugs in this fashion to affect the dietary habits of apparently normal individuals in the population to their ultimate advantage is perhaps a new pharmacological concept, is certainly a pharmacological possibility and may, in the future, become an object of pharmacological research.

Use of Vitamins

It is generally assumed that the dietary intake of our population is adequate to meet its requirements, although amongst the very young and the elderly it is accepted that some vitamin supplementation may be necessary. In consequence, even in the winter when the availability of Vitamins C, and A and D may be decreased and infections are more common, it is not recommended that the daily intake of these vitamins should be increased. The recommended daily intake of Vitamin C in adults is 25–75 mg for prophylactic use (BP, 1963). The recommended dose in the USP is 75 mg daily. There is a wide-spread belief that ascorbic acid in doses of 200–1,000 mg daily may have some protective effects against the common cold. Ascorbic acid is said to produce this protective effect both in virtue of its prophylactic action against the common cold and as a result of a therapeutic effect when the symptoms of the common cold appear. The evidence supporting either of these actions of Vitamin C is at present by no means conclusive, and indeed a leading article in Nutrition Reviews (1967) states that there is no conclusive evidence that, in the absence of severe ascorbic acid depletion, ascorbic acid has any effect on the incidence, course, or duration of the common cold. This conclusion is supported by the evidence of Walker, Bynoe and Tyrrell (1967) from the common cold research centre. Their trial involving administration of 1,000 mg daily for 3 days in its design resembled a therapeutic trial more nearly than it resembled the prophylactic trials carried out by other investigators. The results indicated that ascorbic acid did not have any effect on the experimental colds which were produced in their volunteers. The authors did not state whether they used male or female subjects, and they did not provide a breakdown of the symptoms from which the subjects suffered (Wilson, 1967*b*). The available evidence therefore suggests that Vitamin C does not have any effect on the common cold, and it has been used to argue that any prophylactic effects which ascorbic acid might have are as ineffective as its therapeutic effects. On the basis of this evidence television advertising of the value of Vitamin C for the common cold has been withdrawn, and the conclusion must be drawn that the Vitamin is being abused by the public.

Since 1965 a series of investigations involving the administration of 200 mg daily have been carried out on students and school-children in Dublin in which the effect of Vitamin C has been investigated on the symptoms of the common cold in males and females. The investigations have been designed as prophylactic trials. They have been carried out under double blind conditions and incorporated the administration of identical dummy tablets and tablets of ascorbic acid. The presence or absence of symptoms of the common cold were recorded daily, and in all a total of about six hundred subjects have been investigated during two winter seasons. The results of the investigations were fed into a computer and analysed statistically. They indicated that ascorbic acid has a significant prophylactic effect on the symptoms of the common cold. The origin and implications of this observation are related to diet, and to drug use by the population. Belief in the prophylactic effect of Vitamin C against the symptoms of the common cold has been extant for several years, and has been encouraged by advertisements. The relatively low figure in the B.P. for the dietary intake of this Vitamin in comparison with the American and Russian figures has been accepted since the MRC report in 1953. The mechanism by which ascorbic produces these effects on the symptoms of the common cold is unknown, but the observation that it does produce these effects raises the question as to whether the vitamin under these circumstances is acting as an essential article which is apparently deficient in the diet of our population, or as a drug which is acting as an anti-infective agent.

Insecticides

Drug development, use and control has other implications in regard to the health of human populations, production of their food-stuffs, and preservation of their environments. This is of particular importance in a largely agricultural nation like Ireland where insecticides can have outstanding effects. Fundamental research is at present being

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carried out in the department on the effects of insecticides in mammals and man. In particular the insecticides known to accumulate in the fat of humans and animals are being studied with special reference to their activity in the non-medullated nerves of the central nervous system. It is possible that this work will provide further knowledge of the mode of action of drugs in the brain, and will supply information about the hazards to man which can arise from the entry of this type of pesticide into our environment (Chambers, 1968). Environmental poisoning is not the prerogative of human beings alone. An example of contamination of animal feeding stuff with a pesticide containing dicoumarol has recently been described (Pugh, 1966). Such contamination can potentially give rise to serious agricultural hazards, and cause economic loss if strict controls are not ensured. Naturally occurring disease problems among livestock are of considerable significance in Ireland. Basic research into control of liver-fluke disease is being carried out in the department (Pugh, 1965) as one of the aspects of applied and social pharmacology, since it is recognized that herd dosing on a sample basis is particularly a characteristic of drug evaluation in agriculture. The use, and hazards arising from the misuse, of pharmacologically active compounds in all forms of human, animal and crop treatment and development are becoming increasingly important in our everyday lives and economy. Awareness of this is necessary for our well being, and research into methods for evaluation of the effects which these compounds produce is essential in order that we can be aware of their relative benefits and hazards on a community basis.

Drug Trials

In his lecture Sir John discussed the development, evaluation and testing of drugs, and he described the difficulties and complications which are associated with the analysis of their efficacy among diseased individuals in the population. Sir John said that the final proof which indicated that anti-coagulants do not benefit arterial thrombosis, was based on statistical evidence. The analysis of a number of symptoms in a large population over a prolonged period is an extensive task, and I agree wholeheartedly with Sir John that for investigations on large population samples nowadays it is essential to design the trials so that the number of subjects included, and the duration of the trial, should be such that a reasonable statistical analysis of the results can be carried out.

Developments in pharmacology today emphasize the importance of two general lines of research both of which require concerted and co-operative effort. Dr Bain has pointed out how the pharmaceutical industry, the academic pharmacologists pure and applied, and the drug administrators who are independent of government control, can co-operate to put such concerted effort into effect. The first line is basic pharmacological research. It is directed towards the development of new compounds for the treatment of disease in individuals. The expertise, man-power, and equipment of the pharmaceutical industry enables the investigators in its laboratories to play the leading role in this field of developmental research. However, this is continually becoming more demanding of trained personnel, more technical, and more expensive. Perhaps for this very reason we do not have any extensive pharmaceutical research in Ireland. Even in a relatively small country like this however, the industry cannot afford to use such a reason as an excuse for not attempting to carry out basic research. Basic research has a place here; it can be directed towards improvements in chemical, pharmaceutical and pharmacological methodology; it can extend and better our pharmaceutical engineering; it can investigate the storage and stability of drugs under various conditions, and discover better and more efficient forms of packaging. In addition research into methods and performance of raw material testing could add greatly to the rate of turn-over of pharmaceutical materials and their preparation for export. This is perhaps a more extensive programme of basic research than any one company would care to mount alone. Since, however, it could be of benefit to all Irish companies, perhaps the time has come for the industry to consider whether a combined basic research programme in this field would profit all the companies who agreed to co-operate in putting it into effect.

The second line of pharmacological research which is increasingly becoming of importance is the clinical evaluation of drugs in sample populations, examination of their toxic effects and investigation of drug interactions. Only a few years ago the last was considered to be of minor importance, but now the extensive consumption, and abuse, of drugs by the population at large has drawn attention to the complicated effects of drug interactions arising from many unsuspected causes and among all members of the population. Interactions between common foods in the diet and chemical compounds in the environment, and drugs, adds to the difficulties of analysis of complications which occur, and hazards which arise in the Irish population. The appointment of the National Drugs Advisory Board has provided an opportunity for the control and evaluation of new drugs introduced into the country. However, the clinical investigation and examination of the effects of the pharmacologically active agents being used by the population as a whole, evaluation of their interactions and study of their relationship to

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the national diet, environmental conditions, and genetic characters of the population has hardly been attempted. Research into social pharmacology is still an undeveloped field throughout the world. The opportunities for carrying it out in Ireland are great; the results with regard to the clinical trial of drugs, and benefit to the health of the population could be outstanding.

References

Bartley, W., Krebs, H.A., O'Brien, J.R.P. (1953) *Spec. Rep. Ser. Med. Res. Coun.* (Lond.), No. 280.

Chambers, P.L. (1968) in press, *Proc. Roy. Irish Acad.*

Leading Article (1967) *Nutrition Review*, 25, 228-231.

Pugh, D.M. (1965) *Irish Vet. J.* 19, No. 12.

Pugh, D.M. (1966) Proc. of IV Int. Meeting of the World Ass. for Buiatrics (Zurich).

Rick, J.T. and Wilson, C.W.M. (1966) *Quart. J. Stud. Alcohol*, 27, 447-458.

Walker, G.H., Bynoe, M.L., Tyrrell, D.A.J. (1967) *Brit. med. J.*, 1, 603-6.

W.H.O. 1965. WHO Expert Committee on Dependence producing drugs, 14th Report, Technical Report Series No. 312, World Health Organization, Geneva.

Wilson, C.W.M. (1967a) *Brit. med. J.* 2, 698-9.

Wilson, C.W.M. (1967b) *An Cogaseoir*, 3:4, 13-27.

Reprinted from the BRITISH MEDICAL JOURNAL, 20 *March* 1971, 1, 669

Vitamin C and the Common Cold

(*To the* EDITOR *of the* BRITISH MEDICAL JOURNAL)

SIR,—An article appeared in the *Sunday Times* of 14 February in which Dr. Linus Pauling's book *Vitamin C and the Common Cold*[1] was discussed. During the past six years a programme of research has been carried out in this department on various aspects of the metabolism and action of vitamin C, and several papers have now been published describing the results.[2-7] Included in this programme has been a study on the relationship between the administration of supplementary vitamin C and the occurrence of symptoms of the common cold. Dr. Pauling referred to the results of these investigations in his book.

Any discussion about vitamin C and the common cold must take into account the human requirements for exogenous vitamin C. The United Kingdom Panel on Recommended Allowances of Nutrients points out that there are two theories about the human requirements for vitamin C.[8] One specifies that humans require only sufficient exogenous vitamin C to prevent them from degenerating into a scorbutic state. The other theory proposes that human beings should, ideally, be saturated with vitamin C. They would then physiologically resemble other animals which can make sufficient vitamin C to keep themselves continuously saturated. The official British attitude is to support the former theory. The Americans

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and Russians incline to support the latter theory. Accordingly they specify larger daily human requirements for vitamin C.

It was stated in the *Sunday Times* article that many trials have been carried out in an attempt to investigate the relationship between administration of supplementary vitamin C and control of the common cold. The results of these trials have been reviewed in *Nutrition Reviews*[9] and more recently by Regnier.[10 11] Examination of the results of these trials makes two points evident. The first is that all the trials have measured the relationship between the appearance and severity of symptoms of the common cold and the administration of various doses of supplementary vitamin C for varying periods of time to different population samples. In addition, Dr. D. A. J. Tyrrell's trial[12] attempted to evaluate the relationship between viruses associated with the appearance of common cold symptoms and administration of supplementary vitamin C. In the latter trial, however, production of cold symptoms in subjects inoculated with common cold viruses had a success rate of only 38%. No information was provided about the sex, age, or vitamin C status of the treated or control volunteer subjects.[13] There is considerable evidence that the first two factors affect ascorbic acid metabolism in different individuals, and that the third factor influences tissue integrity and normal function.[14] Nothing can therefore be concluded about the relationship of ascorbic acid metabolism and the appearance of cold symptoms in the different experimental groups from the results of this trial. In the *Sunday Times* it was stated that this trial, carried out at the Common Cold Research Unit, was probably the best conducted up to the present date. It may successfully have demonstrated the relationship between the common cold viruses and development of symptoms of the common cold, but by modern standards of clinical trial methodology it could not be classified as a well conducted clinical trial on the relationship between development of the clinical features of the common cold and the administration of supplementary vitamin C.

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The second point which is apparent from all the clinical trials on vitamin C and the common cold is that no investigations, apart from those carried out in Dublin,[15] have examined the vitamin C tissue status of individuals who have colds. Development of symptoms of the common cold is dependent upon a particular virus-host relationship being attained. Dr. Tyrrell has demonstrated that infection of a host with a suitable virus must be achieved before a cold can develop.[16] However, it is equally necessary for the host's tissues to be in a susceptible state for attack by the virus. As yet no evidence has been published which demonstrates whether this susceptible state is associated with an abnormality of vitamin C metabolism in the host at the time of attack by the virus. It has already been demonstrated that a significant elevation of tissue levels of vitamin C occurs in subjects susceptible to attack by common cold viruses when they are given supplementary vitamin C during the winter months,[15 17] and that this supplementary ascorbic acid significantly reduces the duration, severity, and incidence of common cold symptoms in adolescent subjects.[16] However the final proof of the efficacy of supplementary vitamin C in reducing the severity of common cold symptoms requires critical interpretation of the state of ascorbic acid metabolism in the infected subjects during their colds. Such interpretation is dependent upon accurate knowledge of the relationship between their plasma and leucocyte ascorbic acid values, and the state of their ascorbic acid stores.[18 19]

Dr. Pauling did not provide this critical evidence necessary for support of his hypothesis about the relationship between the administration of supplementary vitamin C and reduction of the symptoms of the common cold.—I am, etc.,

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1 Pauling, L., *Vitamin C and the Common Cold*, San Francisco, W. H. Freeman and Co., 1970.
2 Loh, H. S., and Wilson, C. W. M., *British Journal of Pharmacology*, 1970, 40, 169P.
3 Loh, H. S., and Wilson, C. W. M., *British Journal of Pharmacology*, 1970, 40, 566P.
4 Odumosu, A., and Wilson, C. W. M., *British Journal of Pharmacology*, 1970, 40, 171P.
5 Odumosu, A., and Wilson, C. W. M., *British Journal of Pharmacology*, 1970, 40, 548P.
6 Loh, H. S., and Wilson, C. W. M., *Lancet*, 1971, 1, 110.
7 Loh, H. S., and Wilson, C. W. M., *International Journal of Vitamins and Nutrition Research*, 1971, in press.
8 Passmore, R., *Recommend Intakes of Nutrients for the United Kingdom*. London, H.M.S.O., 1969.
9 *Nutrition Reviews*, 1967, 25, 288.
10 Regnier, E., *Review of Allergy*, 1968, 22, 835.
11 Regnier, E., *Review of Allergy*, 1968, 22, 948.
12 Walker, G. H., Bynoe, M. L., and Tyrrell, D. A. J., *British Medical Journal*, 1967, 1, 603.
13 Wilson, C. W. M., *British Medical Journal*, 1967, 2, 698.
14 Wilson, C. W. M., *Vitamins*, Hoffman-La Roche, Basle, 1971.
15 Wilson, C. W. M., and Loh, H. S., *Acta Allergolica*, 1969, 24, 367.
16 Tyrrell, D. A. J., *Common Colds and Related Diseases*. London, Edward Arnold Ltd., 1965.
17 Loh, H. S., and Wilson, C. W. M., *British Journal of Nutrition* 1971, in press.
18 Loh, H. S., and Wilson, C. W. M., *British Journal of Nutrition* 1971, in press.
19 Loh, H. S., and Wilson, C. W. M., *International Journal of Vitamin and Nutrition Research*, 1971, in press.

Irish J. Med. Sci. 8(3): 345-355 1970

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THE DIETS OF ELDERLY PEOPLE IN DUBLIN

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Summary

A DIETARY survey was carried out between October and December, 1969, on thirty-one elderly healthy subjects aged 64 to 86 years living in central Dublin. Their living conditions in relation to age, sex and social contacts, and in relation to dietary intake were analysed. Eighty-seven per cent lived alone but contact with church, family and friends provided social contact to varying degrees. Just over half the subjects depended on day clubs or Meals on Wheels for supplementary dietary intake. The average dietary intake of the sample was generally adequate, but was below the recommended British Standards for Calories and Calcium. However 36% of the subjects had an intake of Vitamin C, and 6% had an intake of iron, below the recommended British Standards. The intake of nutrients diminished with increasing age. Dietary intake was related to living conditions, the subjects living with relatives having the best intake, and those living alone and dependent on Meals on Wheels having the lowest intake.

Introduction

It has been pointed out by Exton-Smith and Stanton (1965) that the precise nutritional needs of old people are unknown. The recommended intakes for nutrients are defined as the amounts sufficient or more than sufficient for the nutritional needs of practically all healthy persons in a population (HMSO, 1969). It is however known that the intake of individual nutrients influences the requirements of other nutrients (HMSO, 1969) and that differences in activity, sex and age have profound effects on the needs of different samples of the population (Cuthbertson, 1964) Efforts to establish precise nutritional needs of a section of the population therefore come up against the problem of defining the state of optimum health with which the nutritional needs of the particular population should be correlated. Exton-Smith and Stanton attempted to do this by surveying the nutritional intake of elderly women living in London by questionnaire and recipe methods (Schaefer, 1966), and evaluating their state of health by suitable clinical and biochemical examinations. Having established the correlation between nutritional status and state of health in a particular population in this way, it is then possible to find out whether alteration of the nutrition in

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specific ways can improve the state of health. The beneficial effect on health produced by administration of folic acid to pregnant women, (Chanarin, *et al.*, 1968), the improvement in the state of dentition obtained by addition of fluoride to water (Arnold, *et al.*, 1956), the increase in haemoglobin level produced by administration of supplementary vitamin C to elderly subjects (Loh and Wilson, 1970), and the prophylactic-effect of vitamin C on the common cold (Wilson and Loh, 1970) provide examples of the way in which an optimum nutritional status in specific nutrients can give rise to improved health.

Little information is available about the dietary intake of old people in Ireland, although O'Sullivan, *et al.* (1968) have investigated the vitamin C intake of old people in Cork thee-quarters of whom were ill and one quarter of whom were healthy old people. In the course of studies on the vitamin C intake of old people, a survey of the nutritional status of healthy old people living at home in central Dublin was carried out by the questionnaire method. The results of this study are presented here.

Methods

Twenty-eight women and three men were included in the survey. Their ages ranged from sixty-four to eighty-six years. Four had been patients in a Dublin geriatric hospital (M) and the others were selected at random from the membership lists of two Dublin clubs for elderly people. Some of the subjects were found ineligible because of deafness, or mental confusion which would have made communication difficult. All the subjects who finally participated were apparently healthy and mentally alert.

Each subject was contacted individually at his or her home and the purpose of the survey explained. After the initial contact, each subject was interviewed at least twice more. The interviews were conducted at home, except in some cases who spent most of their time at a Club where they were interviewed.

The subject reported the type and amount of food eaten at each meal since the previous visit. Most of the subjects were able to write down the necessary information themselves; in cases of illiteracy or physical handicap, the information was written down by the interviewer. Wherever possible, the help of a neighbour or relative was enlisted. Each subject then completed a form listing all food and beverages taken during the course of the week. It was possible by asking general questions to ascertain whether or not the dietary intake of that particular week seemed to be typical of the subject's general diet. Another form was used to investigate factors in the subject's life, such as living conditions, degree of independence and social activities. This form was completed by the interviewer, who obtained the information either from direct observation or the subject's answers to questions. The diet was analysed for protein, fat, carbohydrate and calorie intake, and consumption of calcium, iron, and vitamin C by the use of the tables prepared by McCance and Widdowson (H.M.S.O., 1960).

Results

The results of the survey are described in relation to the age, sex, and social conditions of the sample, and in relation to the dietary intake of the subjects.

Age, Sex and Social Conditions

The age and living conditions of the subjects are shown in Table 1. Many of the subjects when questioned had difficulty in remembering their exact birth-date, but most knew how old they were. Of the three males, one was married and living with his wife, the other two were living with their children. Six of the subjects did not live alone, three lived with a husband or wife, the other three with younger relatives in a family situation. The remainder were asked how long they had lived alone (Table 1). Six did not know exactly, but had lived alone since the death of their husbands. Four unmarried women had lived alone for thirty-five years or more from the time of leaving their parents. Living alone did not appear to affect the subjects' ability to care for themselves, although this factor cannot be readily isolated from other social aspects. A woman who had been living alone for only two years since the death of her husband reported that she had not yet adjusted to her loss, and that it affected her eating habits. Nearly all mentioned loneliness as one of the main problems of old age. This applied even to those who were socially active in church activities and clubs. The feeling of loneliness was counteracted in four ways which were not mutually exclusive although the subjects tended to prefer and use one method more than the others. It appeared that these methods for maintaining social contact were deliberately sought though it was difficult to determine the extent to which one method rather than another was chosen as a conscious process on the part of the old people. The degree to which they maintained contact socially is shown in Table 2.

TABLE 1

The age-range and living conditions of the subjects included in the sample.

Age range	Number	Home conditions	Duration in Years			
			35	8-25	8	unknown
64-69	7	Alone	4	12	3	6
70-75	12	With relatives	3	—	—	—
76-80	4	With partner	3	—	—	—
81-86	8					

Casual daily contact with neighbours and friends was most important for the majority of the subjects. All except three knew some friends and neighbours, and were visited in their homes. Twenty attended clubs on one or more evenings a week. The clubs were organised by the church and consisted of a woman's club meeting once a week, or a day centre for elderly people meeting every day for a midday meal and afternoon activities. Club attendances varied, but just under half the subjects attended five or six days each week, and just under one-third did not attend any club at all. It was

TABLE 2

Characteristics of the sample in respect of social contact and preparation of food. Figures refer to numbers of subjects in each category. Regular visits indicate visits at least once per week. In relation to club attendances number of visits per week are indicated. Responsibility for the preparation of the subjects' food is indicated with reference to the person or organisation which cooked it, and the subjects' facilities for cooking.

VARIETY AND EXTENT OF UTILISATION

Social/Cooking Characteristics	Number of Subjects				
Social Contact	Church Clubs	No Contact	Daily Mass	Priest Visits	Infrequent Contact
CHURCH	12	1	15	1	2
	Having Relatives	No Family	Regular Visits	Week-ends Only	Infrequent Contact
FAMILY	20	11	14	5	1
	Having Friends	No Friends	Regular Visits		
FRIENDS	28	3	28	—	—
	Attends Clubs	No Clubs	5-6 Visits	3-4 Visits	Once Weekly
CLUBS	20	11	13	3	4
Cooking	Subject Alone	Subject Major Part	Relatives	Meals on Wheels	Day Club
RESPONSIBILITY	6	6	2	2	15
	Rings & Oven	Rings Alone	Kitchen	Living Room	Outside House
FACILITIES	19	12	7	23	1

not clear, in the case of the day centre club, how many of the members attended mostly for social reasons as opposed to reasons of having access to an inexpensive midday meal. Twenty subjects had children and other living relatives; fourteen were visited regularly by their families, received presents and letters, and, in some cases, a certain amount of financial aid. Five regularly spent week-ends with relatives and one woman visited her son in America every three years. All the subjects except one had contact with the church which formed a most important centre for social contact and support in their lives. Fifteen went to Mass every day. Twelve attended clubs connected with the Church, participated in church activities, and went on outings. One woman who was house-bound had regular visits from her priest

The subjects who were most active in clubs also received more visits from relatives and friends, and had more social life altogether. At least eleven subjects had very little social life at all. Those who were socially active were the most welcoming towards the interviewer. The moderately active were hesitant at first, then seemed pleased to have visits. The three subjects who seemed to be most isolated from other people were the most difficult; they displayed, if not hostility, a great deal of nervousness throughout the interviews. The subjects stated that visits from relatives were less frequent than they would have wished. All seemed to prefer spending time with younger people. Most of them enjoyed taking part in the survey, and were pleased that an interest was being taken in them. When there was initial hesitation, it was usually overcome by a clear explanation. One woman thought that her pension would be taken away if she was interviewed, and several were under the impression that if they seemed to be eating too well at home, they would no longer be allowed to attend their clubs for meals.

Most lived in Corporation flats and houses of two or three rooms, with adequate toilet facilities. One woman lived in a block of flats reserved for elderly people and a man lived in the house owned by his daughter and son-in-law. Three of the subjects owned their own homes and one woman owned her house, but lived only in two rooms on the ground floor. Tenants occupied the rest. Twenty-five subjects did all their own general housework; of the remainder, one was house-bound and had a neighbour who came in three times a week to clean. Two of the subjects living with relatives did no housework at all, one did a limited amount but was helped by her grand-daughter and two had home-helps who came in three times a week.

Very little information was obtained about their financial situation. They all received pensions and four mentioned other benefits. Six were getting some financial aid from relatives at home and abroad; three more received occasional cheques for Christmas or birthdays. One subject was receiving rent from lodgers. All subjects used National Aid Services—local dispensary, free turf, bus service. It is possible, however, that some individuals who were eligible for benefits were not receiving them because of lack of information. However, most of the subjects who seemed to be in need of assistance were receiving it. With regard to the relationship between their financial situation and the availability of an adequate diet, it was apparent that the important factor was not the quantity of money which was spent on food, but rather the way in which the available money was expended. Individual subjects in apparently the same financial situation varied greatly in their ability to feed themselves properly.

All the subjects who did their own housework also did errands, with occasional help from neighbours and relatives. Only three women mentioned proudly that they did all their own shopping and housework and relied on no one. Those subjects who lived with relatives or had home-helps did few errands. None had food delivered to their homes, with the exception of milk. For all the relatively active subjects, shopping was not a problem, as most had less than five minutes to walk to a shop. The majority shopped every day or every two days, part of the reason for this was inadequate storage facilities. Only one woman owned a refrigerator. Eight women had only shelves on which to store food, but most of them said that they enjoyed shopping and for many this was the only outing of the day.

The ability of the subjects to prepare their own meals varied considerably. This variation was governed by their interest in food as a major function in their daily life. This in turn affected the existence of adequate facilities for the preparation of food in their dwellings and the extent to which they could, or did, depend on the willingness of friends or relatives or clubs to prepare their food for them. The subjects were encouraged to talk about their likes and dislikes in food, and about their dietary habits in general. Most of them consumed the same kind of food day after day, and they ate food that did not require elaborate preparation. About twelve were conscious of nutritional needs, mentioned advice from doctors on diets and health, and tried to eat what was good for them and avoid food that was fattening.

The facilities for home preparation of food and the extent to which they made use of these facilities are shown in Table 2. Nineteen subjects had adequate facilities in the form of rings and an oven to do their own cooking, but only six used these facilities to the extent of preparing all their own food. Facilities were dependent not only on the availability of an adequate cooker but also on the surroundings for cooking. A ring in the corner of the living room was less satisfactory both psychologically and physically than an oven and rings in a kitchen. Only seven subjects had separate kitchens; the rest had small kitchenettes, or had the cooking facilities in the main living room. One husband and wife had their gas cooker on the landing outside the flat door.

Six subjects did all their own cooking. Another six did most of their own cooking, but also ate meals with relatives, at the club, or at restaurants. These subjects varied greatly in their intake of food and general ability. Two subjects ate all their meals with younger relatives who did the cooking. Their eating habits were above average—they also seemed to have a greater variety of food, and more cooked meals.

Dietary intake

Two subjects received Meals on Wheels five times a week. It consisted of a two-course midday meal delivered to their homes which contained meat or fish, potatoes and another vegetable, and some kind of sweet, rice, stewed fruit or custard. The service seemed to be adequate for the supply of nutrition during the week, but it required that the subject cater for himself over the week-end. In consequence the dietary intake of both subjects was reduced when Meals on Wheels was not delivered. For one subject who was dependent on the service while his wife was temporarily in hospital any daily deficiency was of less importance than for the other subject who was semi-invalid, and for whom a neighbour did house-work and a little cooking. During the week-ends her diet consisted of:

Saturday dinner — 2 slices of bread, tea.

Sunday dinner — 2 slices of bread, tea.

Fifteen of the subjects during the week of the survey ate five midday maels at the club. The menu varied little from week to week. Every meal consisted of four courses:

Soup.

Meat—stewed beef, corned beef, pork, roast beef, fish or eggs on Friday.

Potatoes—roast, boiled or mashed.

Vegetables—peas, cabbage, turnips, parsnips.

Sweet—stewed apples, custard, sago, rice.

Tea and biscuits.

The subjects who ate at the club fell into two groups; those who depended primarily on the club midday meal for adequate nutrition, and those who also cooked for themselves at home.

A small but fairly constant proportion varied their intake several times each week. The food intake consisted of milk, butter, sugar, bread, cake and biscuits, beef, potatoes, green vegetables, and eggs which were eaten three times per week or more frequently by two-thirds or more. Pork was the next most popular form of protein. Rice was used by about half the subjects as a source of carbohydrate. Fresh fruit and salads were eaten by only about 20% of these old people. The average daily intake of protein, fat, carbohydrate, minerals, and vitamin C, during the week of the survey, is shown in Table 3. The intake altered with increasing age, protein and fat

TABLE 3

Average daily intake of nutrients during a survey period of one week. The daily intake of protein, fat and carbohydrate is measured in grams, that of calcium, iron and Vitamin C in milligrams and the energy intake is measured in kilocalories. The average intake of the Dublin sample is indicated as a percentage of the recommended dietary allowance in Great Britain.

	Aged 70 and below	% Recommended Intake	Aged Over 70	% Recommended Intake	% Change with age
Protein	59.1	116	52.5	109	—11
Fat	76.9	—	72.3	—	—6
Carbohydrates	192.8	—	198.6	—	+2
Calories	161.9	79	158.2	83	—3
Calcium	391	78	475	95	+21
Iron	16.5	165	14.5	145	—12
Vitamin C	34.0	113	31.0	103	—9

falling and carbohydrate rising slightly as the subjects grew older. Calorie intake diminished due principally to the fall in consumption of fat, the increase in calories provided by carbohydrate being balanced by the fall in calories derived from protein. The calcium intake increased with age, and the intake of iron and vitamin C diminished concurrently to a similar degree. There were only three males among the thirty-one subjects, and their daily intake was within the dietary intake range of the females. The mean intake of the whole group has therefore been calculated and compared with that of recommended dietary allowances in Great Britain (H.M.S.O., 1969). The intake of the Irish group is expressed as a percentage of the recommended intake for British females (Table 3). Calorie intake was just over four-fifths of that recommended in Britain and protein intake was more than adequate. Mean values for the intake of iron and vitamin C exceeded the dietary allowances of iron and vitamin C recommended in Great Britain. However the range of intake of both these substances had a wide spread (Table 4). Eleven, 36%, of the subjects had a daily vitamin C intake of less than the minimum level of 30 mg. and 6% had an iron intake of less than the minimum intake of 10 mg. Each of those who had a deficient intake of iron were also deficient in Vitamin C. This Irish sample was deficient in its calcium intake. The mean intake of the whole group was 94% of the recommended British value for sedentary females.

TABLE 4

Range of daily intake of Vitamin C, calcium and iron in milligrams in the Dublin sample compared with the recommended intake in Great Britain.

Nutrient	Recommended Daily Intake	Sample Mean Intake	Range of Intake	% Less Than Recommended Intake
VITAMIN C	30	32	18- 56	36
CALCIUM	500	471	204-793	61
IRON	10	148	30-234	6

Those who lived with relatives (Group A) tended to have a slightly better overall dietary intake than the club attenders (Group B), (Table 5). Their calcium was remarkably low, but their intake of iron and vitamin C was high. Their calories were derived from carbohydrates whereas the calories of the club attenders were derived more from fat. The group which lived alone, did not attend clubs and depended to a variable extent on Meals on Wheels, was dietetically more deficient than the other two groups. The was their dietary intake of protein, carbohydrates, fat and calories. Their more frequently the club attenders went to their clubs for meals and better intake of calcium, iron and Vitamin C was up to the standard recommended in Great Britain. If the subjects could supplement the meals they obtained at clubs with other resources including cooking facilities and assistance from relatives and neighbours their nutritional intake was higher. For the subjects in Group C who lived alone, Meals on Wheels proved to be a very necessary dietary supplement. Those who did not receive this supplement were deficient in all forms of nutriment.

TABLE 5

Nutrient intake classified according to living conditions at home. Group A: Subjects living with relatives. Group B: Subjects living alone and attending clubs more than four times per week. Group C.: Subjects living alone, not attending clubs and using Meals on Wheels.

Subject Type	No. of Subjects	Protein (G)	Fat (G)	Carbohydrate (G)	Calories	Calcium (mg)	Iron (mg)	Vitamin C (mg)
A	3	55	65	219	1669	239	17	45
B	20	54	78	188	1597	447	15	32
C	8	47	65	164	1399	420	12	27

Discussion

The methods used in this survey give a general idea of the dietary intake of the subjects, and their basic living conditions which are related to nutrition. Ideally the nutritional appraisal of a population should involve three methods of assessment namely questionnaire, recipe, and food composite analysis. However when these methods are used simultaneously on a population, it has been demonstrated that agreement between them is highly satisfactory, and that nutrient levels agree quite well when considered area by area (Schaefer, 1966). It has been shown that the results for vitamin C intake, when calculated by weighing and estimation from food composition tables, are higher than those obtained after chemical analysis of the food (Disselduff & Murphy, 1968). It has further been demonstrated that theva values for the vitamin C content of foods obtained from the tables of McCance and Widdowson (1960) are higher than the quantities of vitamin C actually consumed in nutritional food as it is served on account of the loss produced by handling, overcooking and service of the food (Platt, Eddy and Pellett, 1964). This almost certainly applied to the subjects who derived their food primarily from Clubs, and Meals on Wheels in this survey. It is probable therefore that the values obtained by the questionnaire represent higher levels of intake of the vitamin than were actually eaten by these subjects. Andrews, Brook and Allen (1966) have demonstrated that the intake of dietary vitamin C and leucocyte ascorbic acid levels are correlated. It is therefore not surprising that low concentrations of tissue ascorbic acid have been reported from sections of the population of old people in Cork and Dublin (O'Sullivan, *et al.*, 1968; Loh and Wilson, 1970).

The sample was small, and in certain ways restricted. Elderly people who were deaf, mentally confused, or otherwise difficult to communicate with were not interviewed. Those who were interviewed had contact with social authorities and other organisations. Thus, if they were in need of assistance, they were already receiving it. All were in relatively good health, mentally alert, and either cared for, or well able to take care of themselves. No cases of severe ill-health due to malnutrition were detected. Many of the subjects were not eating particularly well, but all were eating adequately. The services of Meals on Wheels for invalid and otherwise needy people, and of the club, providing not only a midday meal, but also social activities, served a major purpose and played a valuable dietetic role. The Club members seemed to have a slightly higher level of nutrition than the subjects who did not use any social facilities for the provision of meals. The advantages of the club meals are cheapness, service and preparation specifically for the subjects, and social contact. For the elderly they eliminate the problems of buying the food, cooking and washing-up. The club is most convenient for people in the immediate area; during bad weather, membership decreases. This may lead to a decrease in nutritional intake of the individuals who have any form of respiratory or rheumatic disease or for any other reason are unable to cope with inclement weather.

The nutritional intake of this sample of geriatric subjects living at home in Dublin was adequate to provide sufficient for their nutritional needs, except in the case of calories and calcium, as specified for the requirements in the United Kingdom (H.M.S.O., 1969). The intake of calories, protein and fat was reduced with increasing age in the same way as was reported by Exton-Smith and Stanton (1965) for their sample of elderly women living in London. However the fall in consumption of protein and fat was less pronounced than in the London sample, and the carbohydrate intake increased slightly as the subjects grew older which differed from the findings in London geriatric subjects. Calculation of the percentage calories supplied by the protein consumption under and over the age of seventy showed a remarkable similarity ranging from 13.7 to 12.9% in the two age groups. Exton-Smith and Stanton (1965) found essentially similar values for their age groups and concluded that the quality of the diet varied little in subjects of this age range. The subjects living with relatives and the club attenders in the Dublin sample had a high protein intake by the recommended British standards. Exton-Smith and Stanton (1965) correlated a high protein intake with better than average health. The standard of health in the Dublin geriatric subjects who lived with relatives and who went frequently to their clubs certainly appeared to be good.

The mean intake of vitamin C in the subjects over the age of seventy was just above the required level; it was slightly higher for those below seventy years. These levels of intake correspond to those reported in geriatric subjects by Andrews, Brook and Allen (1966) and by O'Sullivan, *et al.* (1968), when calculated by the questionnaire method, and to those reported by Disselduff and Murphy (1968) when estimated by weighing the food consumed. The mean intake of iron was more than adequate. However analysis of the range of intake of iron and vitamin C among the subjects indicated that deficiency in vitamin C consumption was much more pronounced than deficiency in iron intake. Anaemia of the iron-deficiency type is common among old people (Bedford and Wollner, 1958). If the old people in this sample did suffer from anaemia, it is probable that improvement of their intake of vitamin C would be of more therapeutic benefit than the administration of iron for improving the anaemic condition (Loh and Wilson, 1970). Exton-Smith and Stanton (1965) point out that, of their geriatric subjects six were not appreciably anaemic although they did have a low dietary intake of iron. The mean calcium intake was low in the sample and 61% of the subjects had a deficient intake. Although all the subjects drank milk in varying quantities, their intake of milk was generally inadequate to supply the necessary quantity of calcium. Some of the subjects did complain of joint pains and a few were relatively inactive. The relatively high intake of protein suggests that any osteoporosis from which these subjects may have suffered was not attributable to deficiency in maintenance of the bone matrix (Reifenstein and Albright, 1947). Exton-Smith and Stanton (1965) and Exton-Smith, Hodgkinson and Stanton (1966) failed to implicate either protein or calcium deficiency as a cause of skeletal demineralisation in their group of elderly subjects although they did suggest that deficiency of Vitamin D could have been responsible, and Greenwood (1964) has indicated that deficiency of Vitamin C may play a part in production of joint pains.

Acknowledgments

We are grateful to Dr. L. Godfrey and to the social workers at St. Mary's Hospital and in the Coombe Club for their advice and help during the course of this investigation. We should like to express our thanks to Dr. A. N. Exton-Smith and to Dr. D. L. Scott for their comments and suggestions in the preparation of this paper.

References

Andrews, J., Brook, M., Allen, M. A. (1966). Geront. Clinic, 8, 257.
Arnold, F. A., Dean, H. T., Jay, P., Knutson, J. W. (1956), Public Health Rept. (U.S.). 71, 652, 963.
Bedford, P. D., Wallner, L. (1958). Lancet 1, 1144-47.
Chanarin, I., Rothman, D., Ward, A., Perry, J. (1968). Brit. Med. J. 1, 390-397.
Cuthbertson, D. P. (1964) in Nutrition : a comprehensive treatise, 2, 436-488.
Disselduff, M. M., Murphy, E. LaC. In Vitamins in the Elderly, edited by Exton-Smith and Scott, p. 60-65. John Wright and Sons, Bristol.
Exton-Smith, A. N., Hodgkinson, H. M., and Stanton, B. R. (1966). Lancet, 2, 999.
Exton-Smith, A. N., and Stanton, B. R. (1965). King Edward's Hospital Fund, London. Report of an investigation into the dietary of elderly women living alone.
Greenwood, J. (1946). Medical Annals of the District of Columba, 33, 274-276.
H.M.S.O., Dept. of Health and Social Security (1969). Reports on Public Health and Medical Subjects, No. 120. H.M.S.O., London.
Loh, H. S., Wilson, C. W. M. (1970), submitted for publication.
McCance, R. A., Widdowson, E. M. (1960). Medical Research Council Special Report Series, No. 297. H.M.S.O., London.
O'Sullivan, D. J., Callaghan, N., Ferris, J. B., Finucane, J. F., Hegarty, M. (1968). Irish J. Med. Sc. 1, Seventh Series, 151-156.
Platt, B. S., Eddy, T. P., Pellett, P. L. (1963). A study of feeding arrangements and the nutritional value of meals in hospitals, Nuffield Provincial Hospitals Trust, Oxford University Press, London.
Reifenstein, E. C., Albright, F. (1947). J. Clin. Invest. 26, 24.
Schaefer, A. E. (1966). In Nutrition, A comprehensive treatise. 3, 217-264. Academic Press, London.
Wilson, C. W. M., Loh, H. S. (1969). Acta Allergolia, 24, 367.

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PROCEEDINGS COMMUNICATIONS GESELLSCHAFTSVERHANDLUNGEN

BRITISH ALLERGY SOCIETY

The Summer Meeting of the Society was held at the Royal Society of Medicine, London, W. 1. on 7th. June, 1969.

Scientific Programme

The speakers were: Professor C. M. Wilson (Dublin) on "Ascorbic Acid and Upper Respiratory Inflammation", Mr. R. Donovan (London) on "Immunoglobulins in Nasal Polyp Fluid", Dr. J. Brostoff (London) on "The Diagnosis of Perennial Rhinitis", Dr. G. Taylor (Manchester) on "Nasal Provocation Testing", Dr. L. H. Capel (London) on "Disodium Cromoglycate for Perennial Rhinitis in Allergic Subjects".

K. M. Citron, M. D., F. R. C. P.
Honorary Secretary

ASCORBIC ACID AND UPPER RESPIRATORY INFLAMMATION *by*
C. W. M. WILSON and H. S. LOH, The Department of Pharmacology, Trinity College, University of Dublin, Dublin 2.

Problems of definition of the disease, and of classification of the symptoms, arise in clinical trials concerned with the upper respiratory inflammation associated with the common cold. (Wilson, British Medical Journal, June 1967, 1, 698–699). In this acute inflammation, ascorbic acid is said to produce its protective effect both by virtue of its prophylactic action, and as a result of its therapeutic effect when the signs of the cold appear. Certain criteria therefore must be satisfied regarding the definition of symptoms and design and analysis of the investigations; and in respect of the purpose and dosage of ascorbic acid, if valid conclusions are to be obtained when the effect of ascorbic acid is being assessed in clinical trials on the common cold. Symptoms must be defined and their duration measured; trials must be double blind and their therapeutic or prophylactic purpose must be stated; the sex of the subjects must be specified and their environment should be controlled; the number of subjects and duration of the trial must be such that a reasonable statistical analysis of the results can be carried out; it is desirable that measurements of ascorbic acid metabolism should be performed during the course of the trial in order to correlate changes in the symptoms with the form of therapy administered.

These criteria have been investigated during the last four years, during which 200 mg tablets of ascorbic acid and dummy tablets were administered daily to children in boarding schools during the seven winter months. The presence or absence of symptoms of the common cold, (*Tyrrell* 1965) was recorded daily. The results are now described from one female school containing 103 subjects of whom 57 received ascorbic acid and 46 received dummy tablets.

As a result of computer analysis it was found that the symptoms in all the children could be separated into two unrelated groups consisting of sore throat, headache, feverish and out of sorts, defined as toxic colds; and cold in the head, cough, nasal obstruction and nasal discharge, defined as catarrhal colds. Ascorbic acid reduced the incidence, duration and severity of these symptoms in comparison with those in children receiving dummy tablets. The form of the toxic and catarrhal colds was also significantly altered so that symptom association was reduced in the presence of ascorbic acid. Duration of the symptoms, cold in the head and nasal discharge, in catarrhal colds, were reduced from fourteen to eight days in children receiving ascorbic acid. The girls who had received 200 mg ascorbic acid daily for three months had a level 60 ug/10^{8} cells ascorbic acid in their white blood cells which was significantly higher than in the girls who received dummy tablets. The latter had a level of 42.5 ug/10^{8} cells. Other measurements demonstrated that the plasma values of ascorbic acid is altered in young adults who have symptoms associated with the common cold. It is concluded that the prophylactic administration of ascorbic acid to young adults significantly reduces the intensity of the symptoms, and form of their association, in the common cold. This effect is correlated with a significant elevation in the tissue level of ascorbic acid.

IMMUNOGLOBULINS IN NASAL POLYP FLUID *by* R. DONOVAN, S. G. O. JOHANSSON, H. BENNICH and J. F. SOOTHILL

Nasal polyp fluid and serum concentrations of albumin, α macroglobulin, IgE, IgG, IgA and IgM, polyp histology, and a clinical assessment of reaginicity have been studied in 29 patients with nasal polyps.

The concentrations of albumin and α macroglobulin in the polyps are consistent with poorly selective filtration from serum.

Nasal polyp concentrations of IgE, IgG, and IgA are in excess of that which can be explained by filtration, in most patients, suggesting local production.

Serum IgE concentration is related to the clinical diagnosis of reaginicity, but local production of IgE is not.

Local IgE production correlates with the tendency to sneeze.

Polyps infiltrated mainly with eosinophils have higher relative concentrations of α macroglobulin and less selectivity for molecular size, suggesting greater capillary leakiness, than do those infiltrated mainly with lymphocytes.

THE DIAGNOSIS OF PERENNIAL RHINITIS *by* J. BROSTOFF and G. P. WALSH-WARING, Department of Immunology, Arthur Stanley House, The Middlesex Hospital Medical School, London, W. 1.

754 patients with a history of perennial rhinitis were seen in an allergy or rhinitis clinic which was run jointly by an allergist and an E. N. T. surgeon. Patients were diagnosed as having extrinsic rhinitis when there was a discoverable allergic or other cause for their symptoms; these patients comprised a third of the whole. All had positive skin tests which were relevant clinically. Intrinsic rhinitis is a diagnosis of ignorance, *i.e.,* there is no discoverable cause for the patient's symptoms (*e.g.* vasomotor rhinitis). In spite of this 32 per cent of these patients had positive skin tests which were deemed irrelevant clinically. 40 per cent of the patients with nasal polypi had definite extrinsic factors producing their nasal symptoms, although there was no proof that these were the cause of the polypi.

Nasal obstruction has many causes, some of which are allergic. The 'Typical allergic mucosa' which is pale and moist signifies the presence of oedema only and does not imply an allergic pathogenesis. 75 per cent of patients with nasal obstruction, whether or not a history of deafness was obtained, had abnormal audiograms, greater than 10 db. loss in the low frequency range. Vasomotor rhinitis, which may also present with the symptoms of obstruction is not a diagnosis but a description of a pathophysiological condition with many causes.

NASAL PROVOCATION TESTING *by* G. TAYLOR, Department of Bacteriology and Virology, University of Manchester, Brunswick Street, Manchester, 13.

Because of the lack of complete correlation between skin sensitivity and clinical sensitivity in allergic rhinitis it is considered desirable that a testing system more closely related to the clinical situation should be developed. The value of such a system would be greatest in the unusual or difficult patient or as an indicator of sensitivity in research projects. The major difficulties in nasal provocation testing are the standardisation of antigen challenge and measurement of the response. A method is described in which aerosol challenge is used and the response is measured as change in nasal airway resistance. The latter is determined by continuous measurement of flow-pressure variations between the post nasal space and the anterior nares. Results are presented in a group of pollen sensitive subjects in whom antigen dose—airway resistance responses have been measured. The changes in nasal airway resistance on antigen challenge are also compared with skin sensitivity. The method's value as a research produce is illustrated by studies on the effects of sodium cromoglycate on antigen induced nasal airway resistance changes.

Nutr. Reports ~~Int~~ 4(6): 371-382 1971 9149

THE EFFECT OF SUPPLEMENTARY VITAMIN C AND IRON ON HAEMOPOIESIS IN OLD AGE

by

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ABSTRACT

Supplementary Vitamin C (500 mg daily), slow release iron (105 mg elemental iron daily), or the two forms of medication in combination were administered daily by mouth to three groups of male and three groups of female geriatric subjects. Leucocyte ascorbic acid concentrations and haemoglobin levels were measured in all the subjects at intervals during the fourteen week period of the investigation.

Administration of Vitamin C with or without iron caused significant elevations in leucocyte ascorbic acid concentrations in both sexes within one week. These concentrations diminished in both sexes during the sixth week. Additional iron in the males caused a progressive reduction in the ascorbic acid concentrations. In the females it was associated with a reduction and subsequent increase in ascorbic acid concentrations. Iron therefore creates a greater demand for Vitamin C in elderly females. In the males, iron alone, and iron with Vitamin C, caused elevations in the haemoglobin during the trial period. In the females all three types of medication caused an elevation in haemoglobin levels but the most consistent and uniform rise occurred in the group receiving iron with Vitamin C.

In both sexes, changes in ascorbic acid metabolism preceded or were concomitant with, changes in haemoglobin formation. A significant degree of haemopoietic instability developed in the males during the sixth week and in the females during the eighth week of supplementation. Supplementation with iron and Vitamin C together in males can produce a new improved and stable relationship between ascorbic acid and haemoglobin in ten weeks. Daily supplements of iron and Vitamin C together, while able to produce raised haemoglobin levels in females, do not result in an improved and stable relationship between these elements in the blood within fourteen weeks.

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INTRODUCTION

The assumption is generally made that deficiency of iron intake among normal subjects limits the attainment of an optimal haemoglobin level (1). However there is evidence that Vitamin C can alter iron absorption (2), affect its transport (3), and performs a function in its tissue release (4). Dietary intake of Vitamin C in the elderly is often low (5-8) and it seems possible that the hitherto unexplained variability in the response of anaemic and elderly people to iron administration, and often reported failure of success with iron therapy (1) could be attributable to an underlying Vitamin C deficiency which limits optimal haemoglobin formation. The effect produced by combined therapy with Vitamin C and iron (9) supports the suggestion that such medication can produce haemopoiesis in anaemic patients. In the present investigation therefore the response of combined therapy with Vitamin C and iron in combination has been compared with the effects of administration of iron or Vitamin C individually during a fourteen week period in healthy elderly subjects.

METHODS

The geriatric subjects (M2) were drawn from the wing of a large hospital in Dublin (Table 1). They had all been in hospital for at least three months. Hospital records showed that haemoglobin levels had remained stable during this period in comparison with those found at the beginning of the investigation. The old people were healthy and normal for their age but were not able to take care of themselves adequately at home. They cared for themselves in hospital but all their

TABLE 1

NUMBERS AND AGES OF SUBJECTS (M2) IN THE MALE AND FEMALE GROUPS. DAILY SUPPLEMENTATION WITH IRON AND VITAMIN C TO EACH GROUP

GROUP		NUMBERS OF SUBJECTS		MEAN AGES	
		Male	Females	Male	Females
Fe	Ferrogradumet 105 mg Slow Release Iron	7	8	68	73
C	Vitamin C 500 mg ascorbic acid	5	8	79	73
FeC	Ferrograd C 105 mg Fe + 500 mg Vit C	5	14	68	75

meals were supplied through the hospital kitchen. The content of the diets supplied through the kitchen did not vary during the course of the investigation. Particular attention was directed towards the possibility of temporary variation in the dietary intake of individuals during the period of the trial. Individual dietary variation was not observed at any time. There was no alteration in environmental conditions or routine of the subjects during the trial, apart from differences in the types of drug therapy administered to the groups, which might have given rise to any abnormal stress. The subjects were selected for the investigation during September 1969 and divided into three groups of males and females, each group containing 15 subjects. They received a supply of tablets every fortnight and ward orderlies ensured that the medication was taken daily. Two groups, one from each sex (C groups), received Vitamin C tablets (Abbott) containing 500 mg ascorbic acid daily; two groups (Fe groups) each received a Ferrogradumet tablet (Abbott) daily, containing 525 mg ferrous sulphate in slow-release form equivalent to 105 mg of elemental iron; and the last two groups (FeC groups) each received a tablet of Ferrograd C (Abbott) daily, containing 525 mg ferrous sulphate in slow-release form, together with 500 mg ascorbic acid. Administration of the tablets commenced at the beginning of October after control samples of blood had been removed for analysis between 0930 hours and 1200 hours during the last week of September. The investigation was stopped in the middle of January, 1970, when all the subjects had received medication for fourteen weeks. During the course of the trial a number of subjects dropped out or were excluded for various reasons. Only subjects who had completed the whole trial were included in the final results.

Leucocyte ascorbic acid concentrations were estimated by the method of Denson and Bowers (10) using a Coulter Counter for the leucocyte counts. Leucocyte counts and ascorbic acid estimations were carried out in duplicate. Good correlation was obtained between the duplicates (11). One ml. of blood was placed in a sequestrene tube for haemoglobin estimations by the oxyhaemoglobin method using a Beckman spectrophotometer (12).

RESULTS

Haemoglobin levels, and leucocyte ascorbic acid concentrations, were measured at one week, and later at two week intervals. Individual values for each period and overall means and standard deviations throughout the period of supplementation are shown for the leucocyte ascorbic acid concentrations and haemoglobin levels in Table 2.

Leucocyte Ascorbic Acid Concentrations

Supplementary Vitamin C daily, with or without iron, caused a significant elevation in leucocyte ascorbic acid concentrations at the end of the first week of administration in both sexes. The mean value for the ascorbic acid concentrations during the entire period of supplementation was greatest in the female C group, and least in the

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TABLE II

HAEMOGLOBIN LEVELS AND LEUCOCYTE ASCORBIC ACID CONCENTRATIONS IN THE DIFFERENT GROUPS DURING THE COURSE OF THE TRIAL. HAEMOGLOBIN: g/100 ml BLOOD. LEUCOCYTE ASCORBIC ACID CONCENTRATION: ug/10^8 CELLS

MALES

	Fe Group		C Group		FeC Group	
Week	Haemoglobin	Leuc.AA	Haemoglobin	Leuc.AA	Haemoglobin	Leuc.AA
0	14.7±1.6	26.0±17.1	15.1±1.7	24.6±11.7	14.2±1.0	21.5±11.8
1	14.3±1.9	27.1±16.1	14.3±0.9	40.1±19.8	14.3±0.7	34.6±15.0
2	14.9±1.0	25.5±7.1	15.3±1.5	43.6±8.2	14.9±0.8	46.3±14.9
4	15.0±1.3	23.8±5.8	14.9±1.5	39.1±3.6	14.8±1.1	40.1±6.6
6	15.4±2.1	22.8±12.0	15.3±1.3	31.0±7.6	16.5±2.4	37.8±11.3
8	15.1±1.2	22.6±6.8	14.8±0.8	40.3±9.6	15.1±0.7	40.8±15.4
10	15.3±2.0	23.8±9.0	14.7±1.0	45.0±13.0	15.3±1.0	39.8±16.8
14	15.8±1.2	21.8±3.7	15.3±1.0	47.0±1.9	15.5±1.2	41.6±20.3
Mean 1-14	15.0±1.5	24.3±10.4	15.0±1.2	38.6±12.2	15.1±1.3	37.5±14.2

FEMALES

	Fe Group		C Group		FeC Group	
Week	Haemoglobin	Leuc.AA	Haemoglobin	Leuc.AA	Haemoglobin	Leuc.AA
0	13.4±1.3	24.1±9.3	13.1±1.0	25.4±13.8	13.8±0.9	21.9±11.5
1	13.5±0.6	23.3±9.2	13.5±0.7	49.5±10.8	14.0±1.6	41.4±8.7
2	14.4±1.0	29.0±9.5	13.4±1.4	45.6±17.3	14.1±1.0	44.2±9.1
4	14.4±0.4	22.8±7.5	14.1±0.9	44.6±9.1	14.5±1.4	40.0±6.5
6	15.1±0.8	15.0±6.1	14.0±0.7	37.4±13.4	14.1±0.4	32.8±5.3
8	15.7±1.4	20.8±6.2	14.6±1.4	45.9±9.0	14.7±1.2	32.9±8.1
10	14.2±0.7	21.4±8.5	13.7±1.4	50.8±11.3	14.4±0.7	36.6±3.5
14	15.2±1.4	32.0±24.0	14.3±1.2	49.9±18.6	15.3±1.7	34.9±9.8
Mean 1-14	14.4±1.2	23.5±11.0	13.9±1.1	43.2±14.9	14.3±1.2	35.2±10.8

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female FeC group. The difference between these values was statistically significant ($p < 0.05$). The overall ascorbic acid values for the two male groups were similar, and lay between those for the two female groups (Table 2).

Leucocyte ascorbic acid concentrations fluctuated throughout the course of the trial showing an initial rise during the first and second weeks of administration in all the groups (Fig.1). The male FeC group had the largest, and the male Fe group had very small peaks. Ascorbic acid concentrations suddenly fell during the sixth week in all except the male Fe group in which there was a general tendency to decline throughout the trial. The extent of this fall, and the rate of recovery, were characteristically different in the other five groups. The fall was least in the male FeC group. Its ascorbic acid then rose during the eighth week to a concentration which was maintained during the remainder of the trial. The fall was greatest in the males receiving Vitamin C alone. The concentration did not attain the original peak value again until the end of the trial in this group. The final concentrations in the two male groups were similar at the end of the trial. The female FeC group never recovered from the fall in ascorbic acid concentrations which occurred during the sixth week (Fig. 1b). However, ascorbic acid concentrations resumed their initial peak values after four weeks in the C group. These values were then consistently maintained. The female group which received iron alone showed comparable fluctuations in ascorbic acid concentrations to the other two female groups though the absolute ascorbic acid concentrations were consistently lower throughout the course of the trial. Complete recovery from the fall in the sixth week was delayed for eight weeks in this group. However the ascorbic acid concentration finally did exceed the initial peak which had occurred during the second week.

Haemoglobin Levels

At the end of fourteen weeks, supplementation resulted in an overall mean elevation of the haemoglobin in all groups except the males who received Vitamin C alone (Table 2). During the course of the trial haemoglobin levels fluctuated considerably (Fig. 2). The two male groups receiving iron, or Vitamin C alone, showed a fall in haemoglobin level during the first week (Fig. 2a). The haemoglobin rose fairly consistently during the first four weeks of the trial only in the male FeC group. All the male groups showed peak haemoglobin concentrations during the sixth week. During the following two weeks the haemoglobin level fell significantly in the FeC group. Thereafter the haemoglobin increased during the remainder of the trial in this group. In the other two male groups the peak elevation was considerably less during the sixth week. The Fe group showed a consistent increase from the eighth to the fourteenth weeks of the trial so that a rise in haemoglobin level was evident in this group at the end of the trial. Throughout the course of the trial haemoglobin levels merely fluctuated round the initial pre-supplementation value in the C group.

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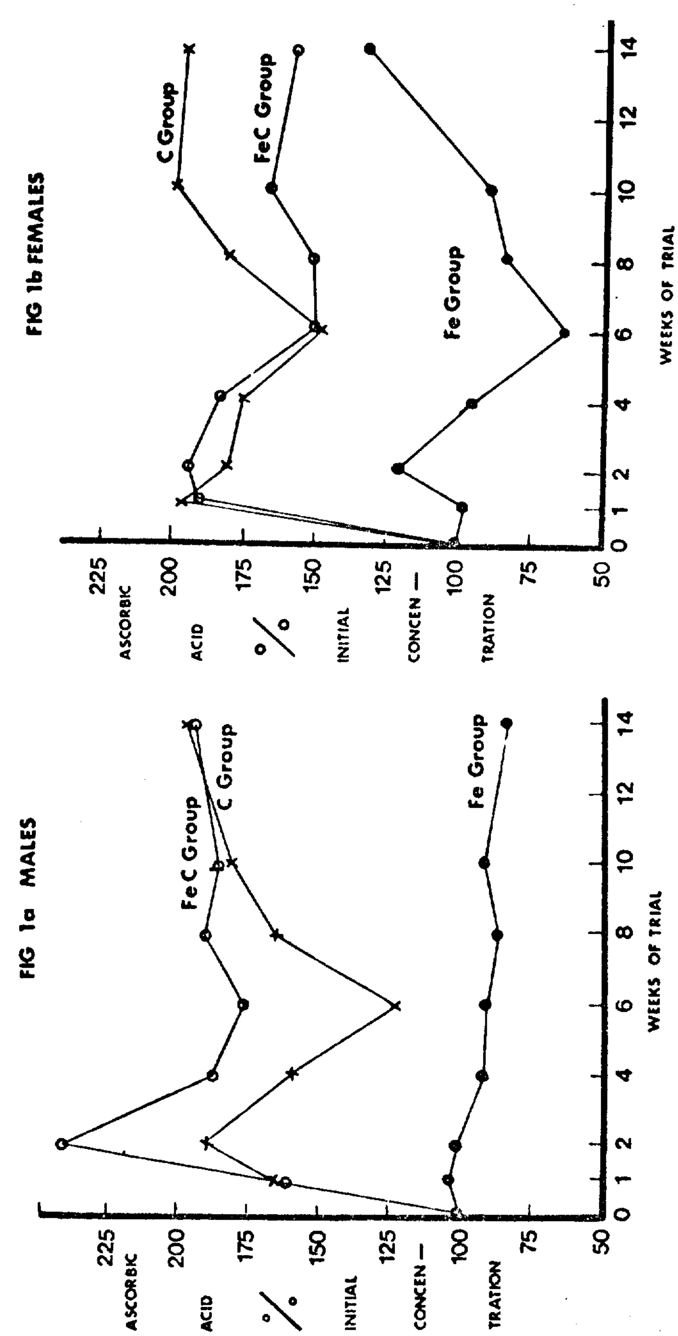

__Figure 1.__ Per cent initial ascorbic acid concentrations in the leucocytes in the groups receiving iron alone (Fe Group o-o), Vitamin C alone (C Group x-x), and iron with Vitamin C in combination (FeC Group o-o).

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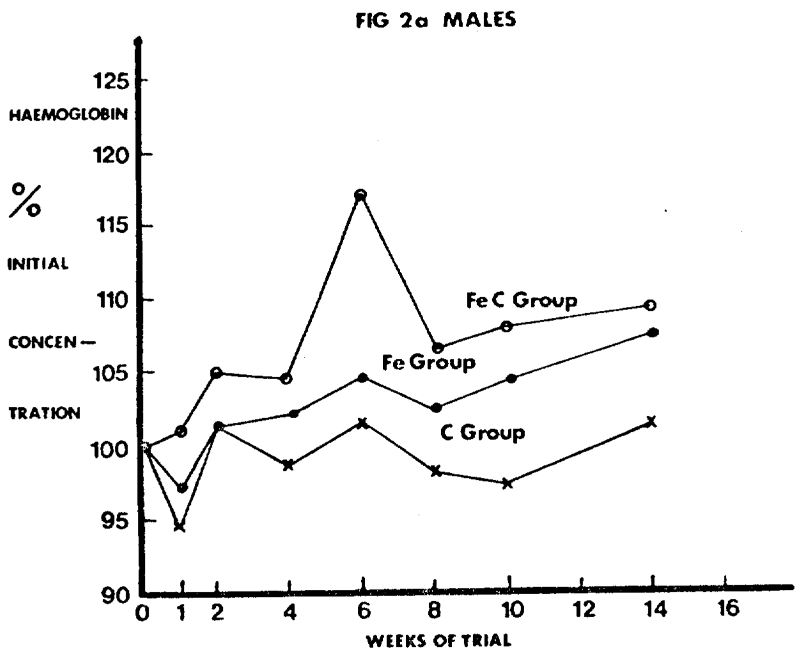

Figure 2a. Per cent initial haemoglobin levels for the groups receiving iron alone (Fe group •-•), Vitamin C alone (C group x-x) and iron with Vitamin C in combination (FeC group o-o).

Haemoglobin rose to a peak level in all the female groups during the eighth week of the trial (Fig. 2b). Up to the eighth week the rise in haemoglobin level was fastest and most consistent in the Fe group. The groups receiving Vitamin C alone, or Vitamin C with iron, showed small falls in haemoglobin levels during the sixth week before reaching their peak levels in the eighth week. All the groups exhibited a sharp fall in haemoglobin levels during the tenth week. This was followed by a consistent and almost parallel rise during the last two weeks of the trial so that haemoglobin levels were similar in all the female groups at the end of the fourteenth week. During the course of the trial the FeC group manifested the most regular increase, and the Fe group showed the widest fluctuations in haemoglobin levels.

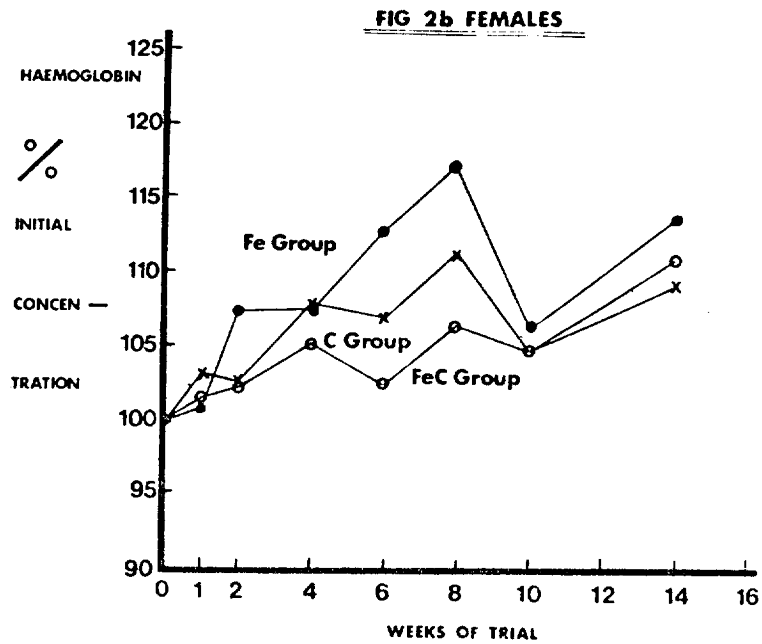

Figure 2b. Per cent initial haemoglobin levels for the groups receiving iron alone (Fe group ●-●), Vitamin C alone (C group x-x) and iron with Vitamin C in combination (FeC group o-o).

DISCUSSION

Administration of 500 mg of Vitamin C daily with or without iron caused a significant elevation in leucocyte ascorbic acid concentrations in both sexes within one week. The mean values for the ascorbic acid concentrations during the period of Vitamin C supplementation were significantly elevated in all four groups. In the two male groups the mean values for the ascorbic acid were similar. Among the males therefore additional supplementation with iron did not influence the overall ascorbic acid concentrations in the leucocytes. However, the overall ascorbic acid concentrations in the two female groups were significantly different during the period of supplementation. The females receiving supplementary iron with the Vitamin C had the lower leucocyte ascorbic acid concentrations. There was no significant difference between the overall ascorbic acid concentrations in the males and females who did not receive iron. It can therefore be concluded that administration of iron results in a greater demand for Vitamin C in elderly females than in elderly males.

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In both sexes leucocyte ascorbic acid reached a peak concentration during the first and second week with all forms of supplementation. The demand for ascorbic acid became evident six weeks after commencing each type of supplementation except in the case of the males receiving iron alone. The heights of the peaks, and the extents and rates at which the demands for ascorbic acid were satisfied, demonstrate the difference between the sexes in ascorbic acid saturation and metabolism, and illustrate the important interdependence of iron and Vitamin C for haemoglobin formation in apparently healthy elderly people. These observations can be explained on the assumption that iron administered by mouth, after absorption in the presence of alimentary ascorbic acid, is used normally by the marrow, but that ascorbic acid is necessary for the mobilisation of iron from the tissues (13). Among the males the ascorbic acid peak was greatest and the demand for ascorbic acid was least in the FeC group. In this group, therefore, the demand for ascorbic acid for the absorption, and mobilisation and use, of iron for haemoglobin formation, was relatively small. In consequence the initial peak was largest in this group and saturation of body stores with ascorbic acid was achieved relatively quickly and easily. In the group receiving Vitamin C alone, the peak was smaller and the demand for Vitamin C was larger. This is understandable because ascorbic acid was in constant demand for absorption, mobilisation, and use of all available iron in this group. Saturation of body stores was achieved temporarily at the beginning of the trial, and again at the end of the trial. In the group receiving iron alone, ascorbic acid was in constant and critical demand. The leucocyte concentrations at the beginning of the trial progressively decreased, and haemoglobin formation was relatively slow. In this male group alone no supply of ascorbic acid was available to meet the demand at six weeks.

Haemoglobin formation and disappearance differed between the sexes in response to the supplementation. Among the males the absence of a fall in haemoglobin level during the first week in the FeC group, and its presence in the other two groups, indicates the necessity of iron and Vitamin C in order to prevent a reduction in haemoglobin level when the stable haemopoietic state is initially being altered. A similar fall in haemoglobin level has been reported during the first week in patients receiving total dose iron infustions with iron dextran (14). A reduction in haemoglobin level has also been demonstrated during the fourth week in patients receiving daily ferrous sulphate therapy (15). This did not occur in the patients receiving ferrous carbonate with ascorbic acid. These observations support the present findings. They indicate the necessity for combined therapy with iron and Vitamin C in order to produce a consistent and uniform increase in haemopoiesis. The increase in haemoglobin during the sixth week, which was greatest in the FeC group, suggests that a haemopoietic surplus occurred during the sixth week. Haemopoiesis suddenly stopped when the ascorbic acid was 91% in the Fe group, 122% in the C group, and 176% in the FeC group, of the initial male values. The surplus was dependent on the availability of iron and Vitamin C

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together, but the Fe group never subsequently exceeded an ascorbic acid percentage of 91%, whereas the percentage in the C group progressively increased from the eighth week onwards. The FeC group maintained a stable ascorbic acid value of about 190% of the initial stable concentration, and resumed normal haemoglobin formation. Haemopoiesis did not occur in the C group because adequate iron was not available to enable haemopoiesis to take place in the presence of the excess ascorbic acid. Throughout the trial haemopoiesis was greatest and most consistent in the FeC group because iron and Vitamin C were available in adequate quantities to ensure controlled haemoglobin formation.

Among the females a small reduction in leucocyte ascorbic acid appeared during the first week in the Fe group. The ascorbic acid concentration increased during the second week as available ascorbic acid was mobilised from body stores and passed into the labile ascorbic acid stores in the leucocytes (16). The available ascorbic acid in the blood diminished after the second week as metabolic requirements for Vitamin C increased in response to the continued iron stress. The rise in blood ascorbic acid concentrations occurred at the same time in the women as the comparable increase takes place in female guinea-pigs on a scorbutogenic diet (17). The lowest value for the leucocyte ascorbic acid, namely 62% of the initial value, was attained in the women approximately two weeks later than the lowest levels for plasma ascorbic acid are attained by female guinea-pigs surviving a scorbutogenic diet. As in the surviving female guinea-pigs in which a readjustment of ascorbic acid metabolism takes place in response to the stress of the scorbutogenic diet, an increase in leucocyte ascorbic acid concentrations occurred in the women in response to the metabolic requirements for ascorbic acid induced by the iron administration. Two weeks after this metabolic readjustment began to occur in the women, a haemopoietic surplus appeared, and haemoglobin synthesis became temporarily arrested. In the FeC and C groups qualitatively similar changes in leucocyte ascorbic acid appeared during the first six weeks to those observed in the Fe group. However the metabolic readjustment in the FeC group was made less apparent by the presence of the exogenous Vitamin C. The most continuous and regular haemopoiesis took place in this group in consequence of the combined effect of the exogenous Vitamin C and the metabolic readjustment of ascorbic acid metabolism induced by the iron stress. A limited increase in haemopoiesis occurred in the C group between the first and second weeks when the ascorbic acid was being used to mobilise tissue iron for haemoglobin synthesis (13). The metabolic readjustment of ascorbic acid metabolism followed at the end of the sixth week, accounting for the increase in leucocyte ascorbic acid concentrations between the sixth and tenth weeks. Further haemoglobin synthesis was then limited in comparison with that which occurred in the FeC group, owing to lack of available iron.

The sex related difference in the utilisation of Vitamin C in humans provides the explanation for the different responses of the sexes to supplementation (16). This can be analysed by relating haemoglobin levels and ascorbic acid concentrations. The males were able to carry out adequate haemopoiesis when they were receiving the full supplement of 105 mg of iron and 500 mg of Vitamin C daily. Lack of either of these haemopoietic factors prevented a complete haemopoietic response in males and resulted in the development of an unstable haemopoietic state and interference with optimal haemoglobin formation (18). Females, in contrast to males, have some capacity to compensate for lack of exogenous Vitamin C during iron therapy by making adjustments in their ascorbic acid metabolism at the end of the sixth week. Administration of iron alone necessitates a more radical readjustment than is required when exogenous Vitamin C is administered with the iron. The haemopoietic response is limited when Vitamin C alone is administered because of lack of easily available iron after the second week. Administration of iron and Vitamin C simultaneously in a ratio of 1 to 5 enables females to make a progressive haemopoietic response. However this combination does not relieve them of the necessity to carry out some metabolic readjustment of their ascorbic acid metabolism at the end of the sixth week. Measurement of the response of the red blood cells alone does not provide adequate information about the state of haemopoietic stability in an individual during administration of iron therapy (16).

ACKNOWLEDGEMENTS

We wish to acknowledge the helpful advice, suggestions and collaboration we have received during the course of these investigations from colleagues in universities and hospitals, and in the pharmaceutical industry. We would also like particularly to thank Mr Paul Dempsey for technical assistance, and Mr P.J. Byrne and Mr Kevin Foran for their help in many ways. We are grateful to Miss Collender and Miss Roberts for their secretarial assistance. We wish to thank Abbott Laboratories for supplies of Ferrogradumet, Vitamin C, and Ferrogradumet C.

REFERENCES

1. Walker, A.R.P. Controversy on iron needs, intake levels, deficiency stigmata and benefits of iron supplementation. Post-grad. Med. J. 45, 747 (1969).

2. Brise, H. and Hallberg, L. Effect of ascorbic acid on iron absorption. Acta Med. Scand. 171, Suppl. No. 376, 51, (1962).

3. Goldberg, A. The enzymic formation of Haem by the incorporation of iron into protoporphyrin: importance of ascorbic acid, ergo thione and gluta thione. Brit. J. Haematol. 5, 150 (1959).

4. Cox, E.V., Meynell, M.J., Northam, B.E., Cooke, W.T. The anaemia of scurvy. Amer. J. Med. 42, 220 (1967).

5. Andrews, J., Brook, M., Allen, M.A. Influence of abode and season on the Vitamin C status of the elderly. Geront Clinic 8, 257 (1966).

6. O'Sullivan, D.J., Callaghan, N., Ferriss, J.B., Finucane, J.F., Hegarty, M. Ascorbic acid deficiency in the elderly. I.J. Med. Science 1 (Seventh Series), 151-156 (1968).

7. Watkin, D.M. Nutritional Problems today in the elderly in the United States. Vitamins in the Elderly (Exton-Smith and Scott, Editors). John Wright & Sons, Bristol, p. 66 (1968).

8. Wilson, C.W.M., Nolan, C. The diets of elderly people in Dublin. I.J. Med. Science 3 (Eighth Series), 345 (1970).

9. Israels, M.C.G., Simmons, A.V. Ferrous Sulphate with ascorbic acid in iron deficiency anaemia. Lancet 1, 1297 (1967).

10. Denson, K.W., Bowers, E.F. The determination of ascorbic acid in white blood cells. A comparison of WBC ascorbic acid and phenolic acid excretion in elderly patients. Clin. Sci. 21, 157 (1961).

11. Loh, H.S., Wilson, C.W.M. An improved method for the measurement of leucocyte ascorbic acid concentrations. Intern. J. Vit. Nutr. Res., 41, 90 (1971).

12. Wintrobe, M.M. Chemical Haematology. Henry Kimpton, London, (1961).

13. Cox, E.V. The anemia of Scurvy. Vitamins and Hormones. Advances in research and applications. (Harris, Wool and Loraine, Editors). Vol. 26, 635, Academic Press, London (1968).

14. Andrews, J., Fairley, A., Barker, R. Total dose in fusion of iron-dextran in the elderly. Scot. Med. J. 12, 208 (1967).

15. Israels, M.C.G., Cook, T.A. New preparations for oral iron therapy. Lancet 2, 654 (1965).

16. Wilson, C.W.M. The metabolic availability of Vitamin C. Vitamins, Hoffman-La Roche, Basle, 3, (1971). In Press.

17. Odumosu, A., Wilson C.W.M. The metabolic availability of Ascorbic Acid in female guinea-pigs. Brit. J. Pharmacol. Proc. (1971) In Press.

18. Loh, H.S., Wilson, C.W.M. The relationship between leucocyte ascorbic acid and haemoglobin levels at different ages. Internat. J. Vit. Nutr. Res. 41, 259 (1971).

Lancet 7497 975-978 illus. 1967

PLATELET ABNORMALITY IN HUMAN SCURVY

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Summary Reduced platelet adhesiveness in two patients with scurvy was rapidly corrected by administration of ascorbic acid. Ascorbic-acid assays demonstrated that normal platelets contain relatively large amounts of ascorbic acid and it is suggested that the defect in platelet adhesiveness may be a factor in the poorly understood hæmostatic defect in human scurvy.

Introduction

WE have measured platelet adhesiveness in response to treatment with ascorbic acid in two patients with scurvy.

Methods

Platelet adhesiveness was estimated by the Hellem technique (Hellem 1960) as modified by Hirsh et al. (1966). Columns, 6 cm. long, containing 2·5 g. Ballotini glass beads 0·57 mm. diameter, were prepared; the main body of the column was constructed of transparent vinyl plastic ('Portex' code no. NT/13, Portland Plastics Limited, Kent), the end-pieces being constructed of 'Esco' translucent silicone tubing (3 mm. bore, 2 mm. wall). Blood was collected by clean venepuncture using a plastic disposable syringe. For platelet studies siliconised glassware was used throughout. 9 ml. of blood were added to 1 ml. of 3·8% sodium citrate which was allowed to stand at least 30 minutes but not more than 45

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minutes before platelet adhesiveness was assayed. 2 ml. disposable syringes ('Plastipak', Becton Dickinson & Co., Ireland) were filled with blood and attached to the Hellem columns, and blood was forced through by means of an electrically driven pump. The time taken for the leading edge of the blood to traverse the whole length of the column of beads was measured. Results varying by more than ±1 second from a mean transit-time of 30 seconds were discarded. The blood issuing from all columns was collected in separate polystyrene tubes. Platelet-counts were carried out in duplicate on blood before and after transmission through the glass bead column.

White-cell counts were carried out using 2% acetic acid as diluent (Dacie 1956). Platelet-counts were performed according to the method of Dacie (1956), using formol citrate as the diluting agent.

Ascorbic acid was assayed by a modification of the method of Denson and Bowers (1961). 3 ml. of blood was added to each of four tubes containing 12·4 ml. of diluting fluid—specimens 1–4. Specimens 1 and 2 stood for 30 minutes at room temperature, red cells being settled by gravitation and 10 ml. of supernatant being separated from each tube; specimens 1 and 2 contained plasma, white blood-cells and platelets. Specimens 3 and 4 stood for 25 minutes at room temperature, centrifugation at 70 *g* for 5 minutes, 10 ml. of supernatant being separated from each tube; specimens 3 and 4 contained plasma and platelets. White-cell counts and platelet-counts were carried out on specimens 1–4. The platelet-counts in specimens 1 and 2 were similar to those in specimens 3 and 4. The white-cell counts on specimens 3 and 4 as compared with those on specimens 1 and 2 revealed that over 90% of the white cells were removed by the centrifugation. All four specimens were then spun at 1600 *g* for 30 minutes; the four pellets were collected, and ascorbic-acid assays were performed on specimens 1 and 2 (white blood-cells plus platelets) and on specimens 3 and 4 (platelets). Ascorbic-acid assays were carried out on plasma from specimens 3 and 4 after high-speed centrifugation; this therefore was a measure of the ascorbic-acid content of the plasma. The details of the preparation of the white-blood-cell platelet pellets differs from that in the method of Denson and Bowers (1961), but the subsequent details were as described by them. The assays were therefore a measure of the content of ascorbic acid in: (1) white blood-cells and platelets; (2) platelets; and (3) plasma (from which white blood-cells and platelets have been removed). Knowing the white-blood-cell count and platelet-count, the ascorbic-acid content could be expressed as µg. per 10^8 platelets, µg. per 10^8 leucocytes, or µg. per ml. plasma.

TABLE I—CASE 1: PLATELET-COUNT, PLATELET ADHESIVENESS, PACKED-CELL VOLUME

Day	Platelet-count (10^3 per c.mm.)	Platelet adhesiveness (%)	P.C.V. (%)
Before treatment:			
1	253	13·6	34
3	231	13·9	..
After treatment with ascorbic acid started:			
3	229	30·4	35
8	182	50·3	36
14	220	60·7	37
16	214	61·9	37
29	181	70·2	42

Case-reports and Results

FIRST CASE

A 55-year-old man admitted to hospital with a history of relatively minor injury to his right leg 2 weeks previously, this being followed by extensive bruising. A few days after the injury and bruising of the right leg, spontaneous bruising of the left leg developed. His diet consisted of white bread, tea, sardines, an occasional slice of meat, and an occasional egg. He disliked fruit and vegetables and had not eaten any of these for at least 2 years. There was no preceding history of a tendency to bruise easily or to bleed excessively. On examination, he had the clinical features of scurvy on the lower limbs with extensive bruising and purpura. Some of the purpuric spots were perifollicular in distribution and there were hyperkeratotic hair follicles. The patient was edentulous and there were no gum changes. There was no doubt clinically that this patient had scurvy.

Platelet adhesiveness was abnormally low (table I). The patient was maintained on a hospital diet for 2 days without ascorbic-acid supplement. A second blood-sample was then obtained which showed adhesiveness essentially unchanged. Ascorbic acid was then administered in a dosage of 700 mg. per day. No new scorbutic lesions developed thereafter and the existing manifestations disappeared rapidly. As shown in table I, after the administration of ascorbic acid, platelet adhesiveness rapidly increased to a high level, at which it was maintained. Throughout the period of study the platelet-count remained relatively constant. The packed-cell volume was constant at 34% throughout the first 13 days of the study, though thereafter it rose. The rise in platelet adhesiveness occurred before the rise in packed-cell volume.

Other investigations giving normal results included blood-urea and serum-electrolytes, liver-function tests, serum-vitamin-B_{12} assay, serum-iron and total iron-binding capacity. The serum-folate was low (3·3 ng. per ml.).

SECOND CASE

A 61-year-old woman admitted with a history of weakness and general malaise, pyrexia, and weight loss. For some weeks she had had an aching pain in the anterior aspect of her legs of sufficient severity to make walking difficult. The patient had been on a very poor diet; in particular she had been taking no fruit, no potatoes, and no other vegetables. There had been very little intake of meat and the main component of her diet had been milk puddings. On physical examination, there was pallor and obvious weight loss. There was hypertrophic gingivitis and extensive spontaneous bruising of the anterior aspect of her legs. Her hæmoglobin on admission was 6·1 g. per 100 ml. and sternal marrow revealed megaloblastic erythropoiesis. The serum-vitamin-B_{12} was normal at 258 pg. per ml., but the serum-folate was low at 2 ng. per ml.

On the history and physical findings a diagnosis of scurvy was justified. This was confirmed by the ascorbic-acid assays shown in table II.

The patient's previous history included treatment for schizophrenia for 2 years as an inpatient in a mental hospital about 14 years before her present admission; at the time of the admission with scurvy there were no psychotic symptoms though there were minor abnormalities of mood. A dietician estimated that the ascorbic-acid content of her diet before admission was 12 mg. per day as compared with a normal of 70 mg. per day; the folic-acid content of her pre-admission diet was estimated at 7 mg. per day as compared with a normal of over 20 mg. per day.

Treatment in hospital consisted initially of ascorbic acid 1 g. per day and a normal hospital diet. Only after a month was folic acid (10 mg. per day) added to the therapy.

As shown in table II the patient's platelet adhesiveness to glass was found to be abnormally low. Table II also shows ascorbic-acid assays, demonstrating that the

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PLATELET ABNORMALITY IN HUMAN SCURVY

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Summary Reduced platelet adhesiveness in two patients with scurvy was rapidly corrected by administration of ascorbic acid. Ascorbic-acid assays demonstrated that normal platelets contain relatively large amounts of ascorbic acid and it is suggested that the defect in platelet adhesiveness may be a factor in the poorly understood hæmostatic defect in human scurvy.

Introduction

WE have measured platelet adhesiveness in response to treatment with ascorbic acid in two patients with scurvy.

Methods

Platelet adhesiveness was estimated by the Hellem technique (Hellem 1960) as modified by Hirsh et al. (1966). Columns, 6 cm. long, containing 2·5 g. Ballotini glass beads 0·57 mm. diameter, were prepared; the main body of the column was constructed of transparent vinyl plastic ('Portex' code no. NT/13, Portland Plastics Limited, Kent), the end-pieces being constructed of 'Esco' translucent silicone tubing (3 mm. bore, 2 mm. wall). Blood was collected by clean venepuncture using a plastic disposable syringe. For platelet studies siliconised glassware was used throughout. 9 ml. of blood were added to 1 ml. of 3·8% sodium citrate which was allowed to stand at least 30 minutes but not more than 45

TABLE II—CASE 2: PLATELET-COUNT, PLATELET ADHESIVENESS, PACKED-CELL VOLUME, AND ASCORBIC-ACID ASSAYS

Day	Platelet-count (10^3 per c.mm.)	Platelet adhesiveness (%)	P.C.V. (%)	Ascorbic-acid assay in: Platelets (µg. per 10^8 cells)	Leucocytes (µg. per 10^8 cells)	Plasma (µg. per ml.)
Before treatment:	416	16·6	. . .	0	16·06	. .
After treatment with ascorbic acid started:						
2	324	31·3	27	4·65	59·92	0·0533
4	283	35·7	26	362·00	58·58	0·1098
9	200	47·5	29	112·80	53·70	0·1111
12	201	49·5	32	272·40	35·40	0·0676
18	217	48·6	. .	142·40	30·60	0·1610
26	212	51·2	37	141·83	38·68	0·0711

patient's platelets seemed to be totally deficient in ascorbic acid, as was her plasma, while the leucocytes contained only 16·06 µg. per 10^8 cells. At the time of this assay the patient had been in hospital for 6 days, being given no therapy other than a normal hospital diet; in fact, she took very little of the food offered to her. She was then treated with ascorbic acid 1 g. per day. The results of the assays on the subsequent samples obtained are shown in table II. Platelet adhesiveness increased significantly (see figure) after administration of ascorbic acid. The distribution of ascorbic acid between the platelets and the leucocytes showed a surprising pattern. While both platelet and leucocyte content of ascorbic acid increased the platelet level rose to over 300-fold the initial pretreatment value, whereas the increase in leucocyte level was only in the order of 4-fold (table II, figure). These high levels were not maintained throughout the period of investigation, but the levels seemed to fall to an equilibrium position in which each platelet possessed four times more ascorbic acid than did the individual leucocyte. After an initial small fall in platelet-count, this remained fairly constant. During the early stages of treatment the packed-cell volume remained fairly constant, an increase only being observed towards the end of the investigation. After administration of ascorbic acid there was a rise in reticulocyte-count to 27%.

Because of the unexpected distribution of ascorbic acid between the platelets and leucocytes which was observed with the above patient, ascorbic-acid assays were performed on blood obtained from 8 healthy adults in approximately the same age-group as the patient. The results are presented in table III. It seems that the major portion of the ascorbic acid in the buffy coat is in the platelets.

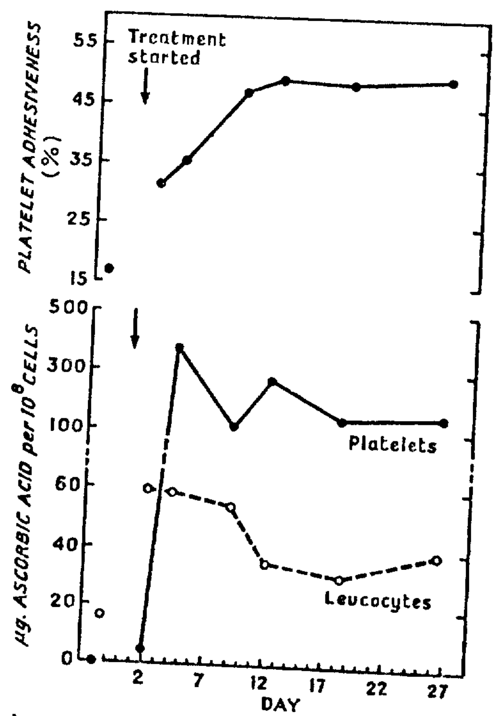
Discussion

Probably the first description of human scurvy was by Le Sieur de Joinville describing the crusade of St. Louis in Egypt in 1260. There followed two centuries later the description of sea scurvy in Vasco da Gama's expedition in 1497 round the Cape of Good Hope to India (Davis 1950). Although this acquired hæmorrhagic disease has been recognised for at least seven centuries, and despite the explosive interest in hæmostasis and hæmostatic failure during the past 25 years, there have been very few reports of demonstrable laboratory abnormalities in human scurvy. We describe here a newly defined abnormality, decreased platelet adhesiveness to glass, in two patients with scurvy; this abnormality is rapidly corrected by the administration of ascorbic acid. It remains to be established whether this is a constant finding in all patients with scurvy.

Previous laboratory studies in human scurvy have been essentially negative. A detailed study of the coagulation mechanism in human scurvy using modern methods has been reported by Hart et al. (1964). All the standard tests of coagulation, including glass activation, gave results which were not abnormal. (One of the patients studied by Hart et al. [1964] had a one-stage test which was 3 seconds longer than the control; this is of doubtful significance.) The platelet-count has generally been reported to be normal. The tourniquet test is usually positive, but may be negative (Hart et al. 1964). Hart et al. (1964), on the basis of examination of blood-films, reported platelet morphology and adhesiveness to be normal, but this represents a very incomplete assessment

TABLE III—ASCORBIC-ACID ASSAYS ON 8 HEALTHY CONTROLS

Control no.	Ascorbic acid (µg. per 10^8 cells) in: Platelets	Leucocytes	Level (µg. per 100 ml.) in platelet-poor and leucocyte-poor plasma
1	60·6	8.5	0
2	52·3	4·8	0
3	24·3	2·9	0
4	87·6	6.0	0·0154
5	150.5	2·5	0
6	122·8	3·2	0
7	96·6	0·9	0
8	91·9	5·6	0
Mean (± S.D.)	85.8(± 40·1)	4·3 (± 2·4)	. .



Case 2: changes in platelet adhesiveness and ascorbic-acid content in platelets and white cells during ascorbic-acid therapy.

of platelet adhesiveness. They found the bleeding-time to be slightly prolonged.

The only report in any detail on the platelets in human scurvy is that of Cetingil et al. (1958), who reported, without giving full technical details, that platelets from a patient with scurvy showed decreased or absent agglutination and adhesiveness when observed by phase-contrast microscopy, and that this abnormality was no longer present on the 10th day after starting treatment with ascorbic acid. They also reported that platelets from patients with scurvy behave abnormally in the thromboplastin-generation test. In a limited similar study of another group of patients with scurvy one of us (A. S. D.) has not been able to confirm this point, and Hart et al. (1964) also found normal results with scorbutic human platelets in the thromboplastin-generation test.

It is customary to estimate ascorbic acid in the buffy coat, which is a mixture of platelets and white cells. The finding of decreased platelet adhesiveness focused attention of the relative ascorbic-acid content of platelets and white cells. The differential estimation of ascorbic acid in platelets and white cells revealed that the platelets accounted for a large proportion of the ascorbic-acid content of the buffy coat. Since the biochemical estimation of ascorbic acid as used by us probably has a large random variation, and since there are inevitable errors in counting of platelets and white cells, and the homogenisation technique is probably short of ideal, assays must be regarded as approximate. However, the differences between white cell and platelet ascorbic-acid levels are so large that the main conclusions regarding distribution of ascorbic acid between platelets and white cells are likely to be valid. The high ascorbic-acid content of human platelets has been previously reported by Barkham and Howard (1958) and by Lloyd et al. (1966). Subsequent to starting therapy in case 2 the ascorbic-acid content of the leucocytes increased tenfold over the mean control value. No final explanation for this is offered; however, during the course of therapy, with this patient, ascorbic acid was detectable in the cell-free plasma, a finding not observed in the control series, and it may be that in the presence of excess ascorbic acid, white cells, and platelets, may be able to absorb and concentrate ascorbic acid.

Hirsh et al. (1966) have shown that platelet adhesiveness is directly proportional to the hæmatocrit (packed-cell volume). We did not consider it necessary to correct for change in hæmatocrit value since in the initial stages of the investigation in which platelet adhesiveness was rising, the packed-cell volume remained relatively constant.

Both patients had folate deficiency and one had a frankly megaloblastic anæmia. Folic-acid deficiency, rather than ascorbic-acid deficiency, may have been responsible for the change in platelet adhesiveness. We investigated two cases of severe megaloblastic anæmia with folate deficiency —one patient had a megaloblastic anæmia of the puerperium and the other patient had anæmia due to the administration of anticonvulsant drugs. The platelet adhesiveness was normal, as were the levels of ascorbic acid in the platelets and white cells.

Our findings were in human scurvy. Important observations have been made in experimental scurvy; decreased platelet adhesiveness in the rotating-bulb method in guineapigs has been reported (Born et al. 1966, Born and Wright 1967). Although Flute and Howard (1959) have shown the plasma of scorbutic guineapigs has a defect of the earliest stages of blood coagulation (i.e., in contact activation) this has not been confirmed in the naturally occurring disease (Hart et al 1964). It may be that experimental scurvy in guineapigs differs in some fundamental way from the human disease.

We thank Prof. E. M. McGirr for his interest in this work and the Medical Research Council for financial support; Dr. G. A. McDonald and Dr. J. F. Davidson for help and for hæmatological data and Dr. E. G. Oastler for permission to investigate case 2. The electrically driven pump was provided by Glaxo Laboratories Ltd.

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REFERENCES

Barkhan, P., Howard, A. N. (1958) *Biochem. J.* 70, 163.
Born, G. V. R., Wright, H. P. (1967) *Lancet*, i, 477.
— — Schneider, M. (1966) *Rep. R. Coll. Surg.* p. 76.
Cetingil, A. I., Alutin, O. N., Karaca, M. (1958) *Br. J. Hæmat.* 4, 350.
Dacie, J. V. (1956) Practical Hæmatology. London.
Davis, L. J. (1950) *Surgo*, 16, 53.
Denson, K. W., Bowers, E. F. (1961) *Clin. Sci.* 21, 157.
Flute, P., Howard, A. N. (1959) *Br. J. Hæmat.* 51, 421.
Hart, H.Ch., Ploem, J. E., Panders, J. T., Verloop, M. C. (1964) *Acta med. scand.* 176, 497.
Hellem, A. J. (1960) *Scand. J. clin. lab. Invest.* 12, suppl. no. 51.
Hirsh, J., McBride, J. A., Dacie, J. V. (1966) *Australas. Ann. Med.* 15, 122.
Lloyd, J. V., Davis, P. S., Lander, H. (1966). Abstracts of papers XIth Congress of the International Society of Hæmatology, Sydney, Australia.

Israel J. Med. Sci. 2: 204-207 1966 9162

VITAMIN C NUTRITION OF ISRAELI INFANTS

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ABSTRACT

Estimation of the vitamin C concentration of breast milk of 64 lying-in mothers showed an average of 6.94 mg/100 ml in May and 4.90 mg/100 ml in November (before the winter citrus season). Five of the 64 specimens showed values below 3 mg/100 ml.

The intradermal dye test for vitamin C was performed on 98 village infants on various diets. Infants receiving cow's milk alone showed a significantly longer duration of the dye than breast-fed infants, breast-fed infants receiving fruit juice and infants receiving cow's milk plus fruit juice. The results suggest that the giving of fruit juice to breast-fed infants did not improve their vitamin C status.

Public health policy may recommend that mothers who breast-feed their infants and have an adequate vitamin C intake do not need to give their infants vitamin C supplements.

The question of prescribing a vitamin C supplement for breast-fed infants in Israel required clarification since breast feeding is common in Israel (1) and since there were no local figures available on the vitamin C content of breast milk (personal communication from K. Guggenheim). Scurvy has been described here but is very rare. Our study included two investigations: A) Determination of the vitamin C content of breast milk during the lying-in period. B) The relation of different types of infant feeding to the vitamin C status of infants as determined by the intradermal dye test (2).

INVESTIGATION A: VITAMIN C CONTENT OF BREAST MILK

Specimens of breast milk were obtained from 64 unselected mothers in the lying-in wards of the Haifa Rothschild Municipal Hospital during the months of May (31 specimens) and November (33 specimens) 1964. The ascorbic acid content of each specimen was determined by a micromethod (3). Details of the mothers and the day *post-partum* of obtaining specimens are shown in Table 1.

RESULTS

The vitamin C content of the breast milk (mg/100 ml) is shown in Table 2. The 31 specimens obtained during May 1964 showed an average of 6.94 mg/100 ml with a range between 3.99 and 11.64 (standard error 1.59). The 33 specimens obtained during November 1964 showed an average of 4.90 mg/100 ml (range 1.34 to 9.44; SE 1.79). Pooled standard 2/3 cow's milk in water with sugar (sterilized hospital formula) given to neonates was examined and showed a vitamin C content of 0.52 mg/100 ml in May and 0.66 mg/100 ml in November 1964. The difference in ascorbic

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TABLE 1. *Details of the 64 mothers (breast milk examinations)*

		May 1964 *31 mothers*	*November 1964* *33 mothers*
Ethnic group	Ashkenazi Jews	18	14
	Sefardi Jews	10	17
	Non-Jewish (Arab and Druze)	3	2
Parity	Primipara	14	8
	2	11	12
	3	2	8
	4	1	3
	6	2	0
	9	1	2
Age (years)	Under 20	2	2
	20 to 29	25	21
	30 to 39	3	10
	Over 40	1	0
Day *postpartum*:	First	2	0
	2	0	1
	3	1	14
	4	12	12
	5	4	2
	6	5	2
	7	4	1
	8	1	1
	11	2	0

acid content of human milk in May and November is significant (0.001 ⟨*P* ⟨0.01).

Using the figure of 30 mg as the recommended daily dietary allowance of vitamin C for infants (4) and the empirical figure of 1 liter breast milk intake, a breast milk content of under 3 mg/100 ml vitamin C represents a suboptimal value. Five of the 64 specimens showed such values, all from November 1964.

TABLE 2. *Vitamin C content of breast milk (mg/100 ml) 64 specimens*

Vitamin C mg%	*May 1964*	*November 1964*
1 to 1.9	0	2
2 to 2.9	0	3
3 to 3.9	1	4
4 to 4.9	5	8
5 to 5.9	0	7
6 to 6.9	7	7
7 to 7.9	12	0
8 to 8.9	5	1
9 to 9.9	0	1
10 to 10.9	0	0
11 to 11.9	1	0
Total number of specimens	31	33
Average vitamin C content	6.94	4.90
Standard error	1.59	1.79

INVESTIGATION B: VITAMIN C STATUS OF VILLAGE INFANTS

Ninety-eight unselected infants aged between six and ten months from the non-Jewish (Arab and Druze) villages of Kfar Kana, Masha-ad and Daliat-El-Carmel were examined during December 1963 and February 1964. The mothers were interviewed in an attempt to obtain a maximum of details on their age, health, parity and diet, as well as on the feeding and general health of their infants. Although detailed information could not be elicited it was found that the number of foods containing vitamin C in these villages was limited. All mothers said they ate the available fruits and green vegetables (citrus, tomatoes, cabbage and lettuce). The maternal diets appeared to be similar judging from these limited replies. Following a physical examination of the infant, 0.075 ml of a 1/1,600 solution of the dye dichlorophenolindophenol was injected intradermally into the forearm and the time determined for the disappearance of the dye (2, 5).

RESULTS

The 98 infants were classified into four groups according to the diet received. Since fluid cow's milk was unavailable in the villages, artificial feeding was based on powdered cow's milk. Fresh citrus juice or tomato juice or both were the only available sources of vitamin C for the infants. Details of the infants, their diet and average time for disappearance of the dye are shown in Table 3.

Analysis of the results of the intradermal dye test showed: 1) There was no significant difference in the duration of the dye (time for disappearance) between the group of breast-fed infants, the group of breast-fed infants receiving fruit juice and the ones receiving cow's milk plus fruit juice. Fruit juice apparently did not improve the vitamin C status of breast-fed infants. 2) Infants receiving cow's milk alone showed a significantly longer duration of dye (i.e. apparently a poorer vitamin C status) than those in the other three groups ($P = 0.05$).

DISCUSSION

Our study of the vitamin C content of human milk was limited to lying-in mothers. Opinions vary on the change in the vitamin C concentration of breast milk as lactation proceeds (5, 6), but mature milk values are usually obtained about the third day of lactation (7). Thus the concentration of vitamin C in the milk of the lying-in mothers studied serves as a guide to the condition later in lactation. Since the amount of vitamin C in human milk may vary seasonably in variation with the amount of the vitamin in the mother's diet, it is understandable that the values in November, before the citrus season, are lower than in May, just after the winter citrus season, and that the five mothers with the lowest concentration were all tested in November. The results of our examinations of breast milk suggest that the amount of vitamin C secreted by the large majority of mothers during early lactation is adequate for their infants.

American investigators including Macy and Kelly (8) found a vitamin C content of human colostrum (one to five days *postpartum*) ranging between 4.7 and 10.4, with a mean of 7.2 mg/100 ml, and of transitional milk (six to ten days *postpartum*) ranging between 4.5 and 9.0, with a mean of 7.1 mg/100 ml. Friedman and Jolliffe (9) state the average vitamin C content of human milk during the early weeks of lactation to be 5 mg/100 ml. The vitamin C content of breast milk was found to average 2.4 mg/100 ml in India (10), in England during the years 1944–45 to range between 3.2 and 4.0 mg/100 ml and in Germany during 1946–47 to range between 3.2 and 4.3 mg/100 ml (11).

Despite the limitations and inaccuracies of the intradermal dye test (5), the fact that fruit juice supplements to breast-fed infants

TABLE 3. *Details of 98 village infants (dye tests)*

Group no.	*Diet*	*No. of infants*	*Average time for disappearance of dye (min)*	SE
I	Breast milk alone	24	18.3	10.6
II	Breast milk plus fruit juice	42	18.8	8.5
III	Cow's milk plus fruit juice	18	20.7	11.0
IV	Cow's milk alone	14	35.6	14.9

did not yield results significantly different from those receiving only breast milk, is striking. This tentatively suggests that wholly breast-fed village infants in Israel receive adequate vitamin C. Thus the two discrete investigations in this paper both support the contention that most breast-fed infants in Israel do not require extra vitamin C.

Some authors regard the minimum requirement of vitamin C for healthy infants as 8 to 10 mg per day (12). The exact significance of a suboptimal intake is difficult to assess, but the possibility of important subclinical vitamin C undernutrition must be considered (13).

Although the vitamin C concentration of breast milk varies with the mother's intake, there is increasing evidence that, in the absence of an adequate maternal intake, the mother may enter a negative vitamin C balance, secreting more vitamin C in her milk than she hêrself consumes (11). A recent WHO committee advised from 50 to 150 mg ascorbic acid as the recommended daily allowance for nursing mothers (14).

The logical routine to ensure an adequate vitamin C intake in the breast-fed infant is for the public health nurse to stress that the mother herself eat fruit and green vegetables. This simple measure is convenient for the mother and will maintain both mother and infant in an adequate vitamin C nutritional status.

eceived for publication 22 October 1965

Ve thank Drs. Z. Polishuk and A. M. Davies, Mrs. I. Dar and the physicians, nurses and village officials for their kind help. Thanks are also due to Sonol-Israel and the Haifa Pediatric Society for their support. The investigation on the intradermal dye test was performed by S. M. as part of his public health study within the framework of the Hebrew University–Hadassah Medical School.

REFERENCES

1. THAUSTEIN, J., HALEVI, H. S. and MUNDEL, G. Infant feeding practices in Israel. *Pediatrics* **26**: 321, 1960.
2. SLOBODY, L. Intradermal test for vitamin C subnutrition. *J. Lab. clin. Med.* **29**: 464, 1944.
3. NATELSON, S. "Microtechniques of clinical chemistry," 2nd edn. Springfield, Ill., Charles C. Thomas, 1961, p. 120.
4. Recommended dietary allowances. Nat. Acad. Sci. Nat. Res. Counc. Publ. 1146, Washington, 1964.
5. BICKNELL, F. and PRESCOTT, F. "The vitamins in medicine," 3rd. edn. London, Heinemann, 1953, p. 465.
6. PLATT, B. S. and MONCRIEFF, A. Nutritional comparison of human and cow's milk for infant feeding. *Brit. med. Bull.* **5**: 1109, 1947.
7. CLEMENTS, F. W. "Infant nutrition: Its physiological basis." Bristol, Wright, 1949, p. 113.
8. MACY, I. J. and KELLY, H. J. Human milk and cow's milk in infant nutrition, in: Kon, S. K. and Cowie, A. T. (Ed.), "Milk: the mammary gland and its secretion," v. 2, New York, Academic Press, 1961, p. 269.
9. FRIEDMAN, G. J. and JOLLIFFE, N. "Clinical nutrition," 2nd. edn. New York, Hoeber, 1962, p. 661.
10. DEODHAR, A. D., RAJALAKSHMI, R. and RAMAKRISHNAN, C. V. Studies on human lactation, III. *Acta paediat. (Uppsala)*. **53**: 42, 1964.
11. GUNTHER, M. and STANIER, J. E. Studies of undernutrition, Wuppertal 1946–9. *Spec. Rep. Ser. med. Res. Coun. (Lond.)* no. 275, 1951, p. 379.
12. GELLIS, S. S. and KAGAN, B. M. "Current pediatric therapy." Philadelphia, Saunders, 1964, p. 7.
13. MUNDEL, G., FISHL, J. and VARSANO, D. Malnutrition in infants in Israel. *J. Trop. Pediat.* **7**: 23, 1961.
14. Nutrition in pregnancy and lactation. *Wld Hlth Org. techn. Rep. Ser.* **302**: 50, 1965.

Lancet, 1970, i, 1347-1348

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ASCORBIC-ACID TREATMENT FOR OSTEOGENESIS IMPERFECTA

SIR,—We are interested in the possibility that nutritional factors may be useful in the treatment of collagen diseases, and we should like to report the results of a trial of ascorbic acid in osteogenesis imperfecta.

Osteogenesis imperfecta is a metabolic bone condition in which the protein matrix of the bone is believed to be the site of the abnormality.[1] Collagen, the chief component of the matrix, is unique in containing a high proportion of hydroxyproline, which is formed in vivo by hydroxylation of proline. Oxygen, ferrous iron, α-ketoglutarate, and ascorbic acid are required cofactors for the proline-hydroxylase enzyme,[2,3] Urinary excretion of hydroxyproline is regarded as an indicator of collagen metabolism in the body. In animals made scorbutic, hydroxyproline excretion decreases.[4] In contrast, when normal people have been made scorbutic, excretion has been shown to increase.[5] This increase in human beings may represent turnover of partially hydroxylated collagen precursors or increased degradation of collagen.

Ten patients with osteogenesis imperfecta, aged 5–27 years, were studied for 12–17 days in the clinical study centre of The Children's Hospital, Columbus, Ohio, and subsequently followed at home. The patients had histories of total fractures ranging from 4 to over 250. Four normal control subjects were selected, representing the two predominant age-groups. They followed the same dietary regimen, but were not admitted to hospital. A low hydroxyproline diet was given throughout the study, and after an initial 4-day period, 1 g. of L-ascorbic acid was given daily.

Ascorbic-acid retention, calculated as intake minus urinary excretion, ranged from 255 to 612 mg. after 3 months. In the young controls, retention also remained relatively high, but a much smaller retention was observed in the older controls. A decrease in urinary hydroxyproline excretion was observed after the 3-month period in six of the ten patients. It may be postulated that there was increased deposition of more stable collagen or decreased collagen degradation. Further evidence of a change in collagen metabolism is a decrease in the incidence of broken bones. At the previous rate of breaks, 20–22 fractures could have been expected in the time period since the start of the ascorbic-acid supplement. Actually, only 5 were reported. 2 of these resulted from substantial trauma; the other 3 were typical of the disease. This decrease in the number of breaks is dramatic, and indicates that this approach is promising for the treatment of this, and possibly other, collagen diseases. That the approach

1. McKusick, V. V. Heritable Disorders of Connective Tissue; p. 233. St. Louis, 1966.
2. Udenfriend, S. *Science, N.Y.* 1966, **152**, 1335.
3. Hutton, J. J., Tappel, A. L., Udenfriend, S. *Biochim. biophys. Res. Commun.* 1966, **24**, 179.
4. Martin, G. R., Mergenhagen, S. E., Prockop, D. J. *Nature, Lond.* 1961, **19**, 1008.
5. Burkley, K. *Am. J. clin. Nutr.* 1969, **22**, 547.

is feasible is shown by the ease and inexpensiveness of carrying out the study, and by the absence of complications.

This work was supported by the Clinical Study Center, The Children's Hospital, and United States Public Health Services grant no. 00078.

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Arch. Geschwulstforsch. 1970,35(1), 36-43

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Relations between the Induction of a Skin Carcinoma and the Collagen Content of the Skin and the Synthesis of Ascorbic Acid in Triturus Cristatus

By G. Wirl and F. Seilern-Aspang

With 4 Figures

(Submitted: August 6 1969)

Introduction

The checking of the proliferation and differentiation of the epidermis during the embryogenesis (3,5,23) and regeneration - both reparative and physiological (1)- in vertebrate animals depends on deeper layers of tissue, i. e., the dermis. In the dermis the collagen seems to be of primary importance, probably due to its fibrous construction (7,24,25).

When cancerization of the skin occurrs with dedifferentiation and uncontrolled proliferation of the cells of the epidermis, defects in the dermis are repeatedly taken up as first phase of a malignant growth of the epidermis. One of the skin carcinomas occurring in yearly cycles in the newt (16,17,19) gave the possibility of examining the behaviour of the dermis parallel to the frequency of malignant growths in the epidermis. During the summer month it was impossible to induce skin carcinomas through wounding or introduction of carcinogens, but in the autumn inducibility increased and reached 100 % in January, February and the beginning of March. In this time of year there also appeared many spontaneous carcinomas. The condition was that the animal be prevented from hibernation by being kept at room temperature. In the monthe of April and May the inducibility of carcinoma disappeared completely. On the contrary, a healing began through a total hornification process in the animals with small skin growths.

Histological examinations of skin lesions showed an decrease of granulation tissue in the remaining epitheliazation towards winter. In the winter months no granulation tissue formed after injury to the skin. Wounds of this kind led to carcinomas: after epitheliazation of the wounds the cells of the epithelium infiltrated the underlying tissues, mostly muscular, from which they were not separated by any connective tissue structure. Malignant growth began only after destruction of the dermis, even when the epidermis was not thereby injured. Wounds of the epidermis without injury to dermis did not lead to carcinomas. If immature connective tissue from a regeneration site in a wound, which can form no more regenerative tissue, was implanted, the dermis was regenerated -- no epidermal growths began. In these animals kept over the winter the formation of granulation tissue was renewed again in the spring, and it was impossible to induce carcinoma by wound formation. On the contrary, infiltrations of skin growths that originated in the winter wree encapsulated in the spring by newly appearing connective tissue, and the epidermal growths became completely horny. (16, 17)

A clear connection was thus shown between the appearance of skin carcinomas and the capacity to form connective tissue. This agrees entirely with the before-mentioned finding of development physiology that in embryogenesis and regneration the proliferation and differentiation of the epidermis is checked by connective ~~x~~tissue structures.

Since the formation of connective tissue depends largely on collagen synthesis, the collagen content of the skin in two of the newt strains in the yearly cycle was investigated. Both strains got carcinomas in the above described period during the winter. Ascorbic acid is necessary for the hydroxylation of prolines in the formation of a typical hydroxyproline-containing collagen in vivo (6). Wound-healing processes are greatly disturbed in rodents suffering from scurvy. In reviewing the principal restrictions on carcinoma formation and loss of connective tissue the ascorbic acid content of the kidneys was therefore examined.

Materials and Methods

The experiments were carried out on Triturus cristatus dobrogicus from the region of Neusiedlersee and on Triturus cristatus carnifex from Rumania. The animals were kept in flat basins, 10 and 15 to each, through the whole year at room temperature. Once a week they were fed with Tubifex. The water was changed every 2 days. The experiments carried out were divided between male and female, young animals and adult animals. As no worthwhile differences were observed, these groups were not separated in succeeding experiments.

1. Ascorbic Acid Content of the Kidneys:

The kidney was used for these investigations because the urodeles synthesize ascorbic acid in this organ (14), i.e., they are independent of the ascorbic acid taken up from the food. The kidneys of 3-4 animals were cleaned of clinging tissue fluids with filter paper, weighed and homogenized in trichloroacetic acid. When settled the ascorbic acid was determined by the photometric method of J. H. Roe and C. A. Kuether (13). This method is based on the reaction of the ketone group of ascorbic acid with 2,4 Dinitrophenylhydrazine in acid at 37° C. The ascorbic acid content was obtained by wet weight.

2. Collagen Content of the Skin:

From both sides of the animal, about midway between the front and hind extremities, smooth pieces of skin of diameter = 0.9 cm were taken by means of a round punch. The attached muscles were removed and the collagen transferred to water-soluble gelatine by the method of D.J. Prockop, S. Udenfried and S. Lindstedt (12). The hydroxyproline content was determined by the photometric method of J. F. Woessner jr. (26).

The hydroxyproline content of the pieces of skin was related to their area.

Results

1. The Annual Rhythm of Collagen Content of the Skin

Figures 1 and 2 show the collagen content of the skin of animals caught in spring and autumn. The collagen content of the skin according to these investigations varies exactly contrary to carcinoma susceptibility. In spring and summer there is a maximum of collagen. At the beginning of winter the collagen content declines strongly and reaches its low point at the end of January to February and the beginning of March, thus at the time when the inducibility of Carcinoma is optimal and when spontaneous carcinomas also occur. From the end of March to April and the middle of May the collagen content rises again steeply. Here we also show the decline of carcinomas and the healing of small epidermal growths.

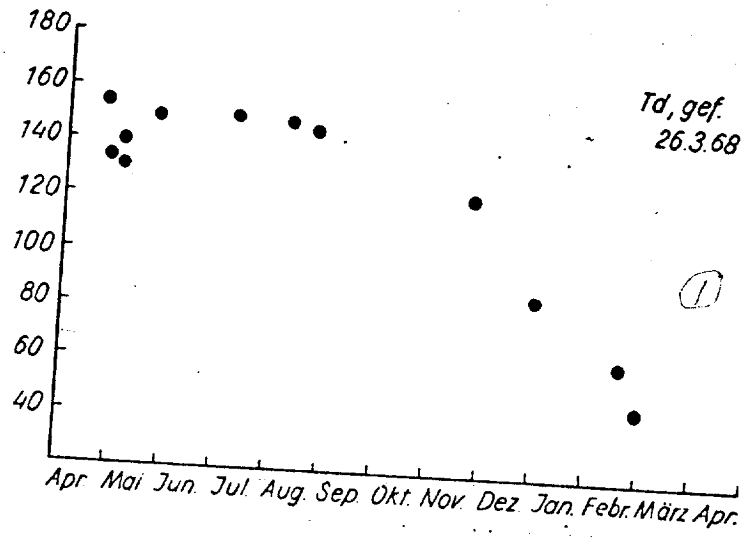

Fig. 1. Collagen Content of Skin of Triturus cristatus dobrigicus Kept All Year at Room Temperature.
gef.= Date of Capture
• Average of 4 tests of 2 Experimental Animals
μ g Hypro/Skin Surface

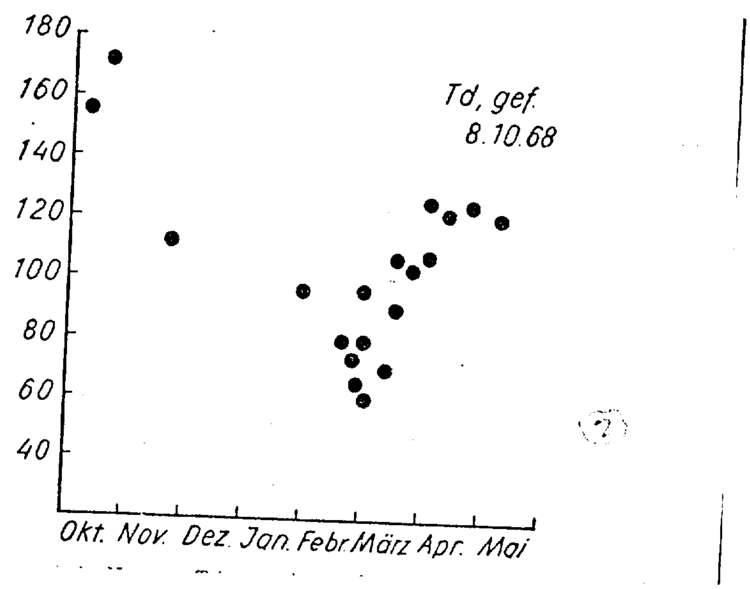

Fig. 2. Collagen Content of Skin of Triturus cristatus dobrigicus kept during Winter at room temperature.
gef.= Date of capture
• = Average of 4 testsof 2 Experimental Animals
μ gH/F = μ g Hypro/Skin Surface

2. The Annual Rhythm of Ascorbic Acid Content in the Newt Kidney

Figures 3 and 4 show the annual cycle of the two strains investigated: soon after capture there follows and increasing loss of ascorbic acid. In the winter months it is no longer detectable, only in the month of March does the production of it rise again. Captures of animals in the wild at various times show that the loss does not occur in the wild.

Discussion

Our studies of the two strains of newts kept for a full year in the laboratory show four occurrences in a striking relationship. None of the four phenomena occurs in wild newts:

1. In the second half of summer the ascorbic acid curve falls. From October to the middle of March no ascorbic acid is detectable. From March it rises again to a largely normal curve in May.

2. Collagen formation, because of the hydroxylation of prolines, depending in vivo on ascorbic acid, follows the synthesis of ascorbic acid in the newt skin. The suddenly appearing decline of it during laboratory holding is visible in its influence on collagen only 3 to 4 months later, due to the slow turnover of the collagen structure.

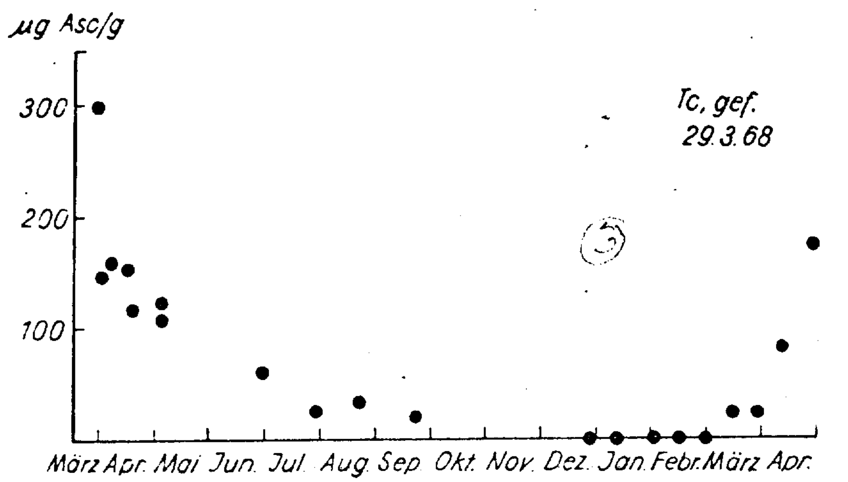

Fig. 3. Ascorbic Acid Content of Kidney of Triturus cristatus carnifex Kept All Year at Room Temperature.
gef.= Date of Capture
● = Average Value of kidneys of 3 to 4 Experimental Animals
µ g Asc/g= µ g Asc/g kidney

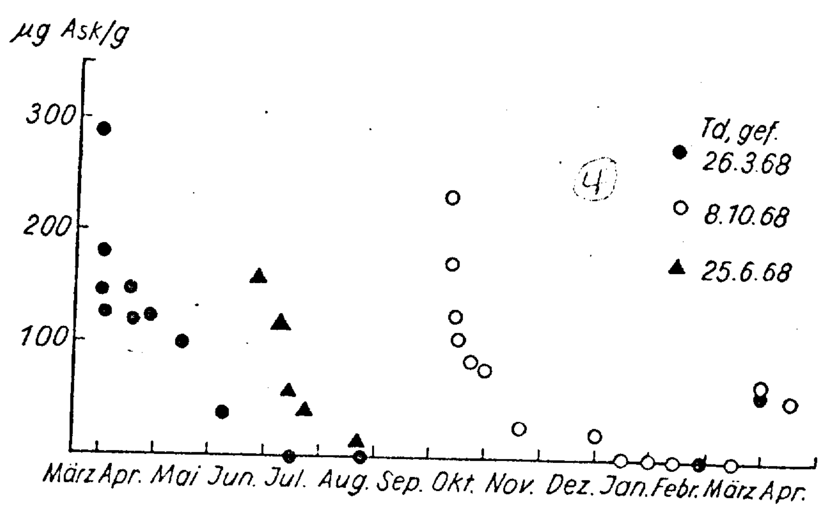

Fig. 4. Ascorbic Acid Content of Kidney of Triturus cristatus dobrigicus. Experiments began at different times with wild captures.
gef.= Date of Capture
● ○ ▲ = Average value of kidneys of 3 to 4 Experimental Animals
µ g Asc/g= µ g Asc/g Kidney

3. As to be expected, after wounding the formation of granulation tissue occurs in a quantity parallel to the curve of collagen synthesis. In the time span when little collagen is found in the newt's skin, there is also no formation of granulation tissue in the skin wounds.

4. The development of skin carcinomas from skin wounds follows ascorbic acid production, inversely with collagen content of the skin and the formation of granulation tissue in wounds.

The increase of inducibility of carcinomas by wounding occurs parallel to the decrease of collagen in the dermis in mouse skin likewise. There also a strong decrease of collagen content (10, 20, 21) follows after application of a carcinogen. Exactly as in newts one can hasten the induction of carcinomas in these zones by wounding (9). Also in transplantation of tail skin into the back of the mouse the collagen content in the transplant sinks almost as strongly as in treatment with carcinogens (22). In such implants one can obtain carcinomas by painting the wounds with benzol. On the other hand there does not occur on the tail of the mouse after treatment with carcinogens either a decline of collagen or a malignant skin growth. All these examples show that in different vertebrate animals there exists a relationship between the development of epidermal carcinomas and the collagen content of the dermis (4, 8, 10, 20, 21).

Since the collagen synthesis depends on the ascorbic acid content its absence could have a cancer-forming influence. Indeed, in outbreaks of carcinomas a decline of ascorbic acid has oftent been observed (2, 11, 15). When we consider the earlier-mentioned significance of the connective tissue structures for the guiding of the proliferation and differentiation of the epidermis in normal growth, it appears to us that the here-occurring defects of collagen synthesis in the dermis standx in a causal relationship to the uncrontrolled (malignant) epidermal growth after wounding.

Summary

1. In a year-long detention of two strains of newts in a laboratory the quantitative occurence of skin carcinomas (spontaneous or experimentally induced by wounding) takes the form of a curve whose minmum is in spring and early summer. The curve rises during the autumn and reaches an optimum in February. At the end of March there follows a sharp decline to the minimum of the curve.

2. In an exactly opposite way behaves the curve of ascorbic acid content of the kidneys (where the urodeles synthesize ascorbic acid), the mass of granulation tissue in the wounds and collagen content in the skin.

3. Since in embryogenesis and regeneration the proliferation and differentiation of the epidermis is controlled by the dermis, specially by the collagen structure, it may be that the deficiency of ascorbic acid and the associated decrease of collagen synthesis because of loss of complete dermis regeneration after wounding, leads to an unctonrolled growth of the epidermis.

Literatur

1. Billingham, R. E., and Silvers, W.: Studies on the conservation of epiderma specifities of skin and certain mucosas in adult mammals. J. Exper. Med. 125 (1966) 429—446.
2. Bodansky, O.: Concentration of ascorbic acid in plasma and white blood cells of patients with cancer and non-cancerous chronic disease. Cancer Res. 11 (1951). 238.
3. Cairns, J. M., and Saunders jr., J. W.: The influence of embryonic mesoderm on the regional specification of epidermal derivatives in the chick. J. Exper. Zool. 127 (1954), 221—248.
4. Gillman, T., Penn, J., Bronks, D., and Roux, M.: Possible significance of abnormal dermal collagen and of epidermal regeneration in the pathologenesis of skin cancer. Brit. J. Cancer 9 (1955), 272.
5. Gomot, L.: Interaction ectoderme — mesoderme dans le formation des invagination uropygienne des oiseaux. J. Embryol. exper. Morph. 6 (1958), 162—170.
6. Gould, B. S.: The Role od Ascorbic acid in Collagen Biosynthesis. In: "Treatise on Collagen" Vol. 2, part A, ed. B. S. Gould, London, New York: Academic Press (1958), p. 323.
7. Grobstein, C., and Cohen, J.: Collagenase: Effect on the morphogenesis of embryonal salivary epithelium in vitro. Science 150 (1965), 626—628.
8. Hamer, D., and Marchant, J.: Collagen and other constituents in the skin of normal carcinogen-treated and castrated mice. Brit. J. Cancer 11 (1957), 445—451.
9. Mac Kenzie, I., and Rous, P.: The experimental disclosure of latent neoplastic changes in tarred skin. J. Exper. Med. 73 (1941), 391—416.
10. Mazzucco, K., Seilern-Aspang, F., und Christian, I.: Unterschiede in der Reitung des Kollagens zwischen Rücken- und Schwanzhaut von Mäusen im Hinblick auf die unterschiedliche Tumoranfälligkeit dieser Hautanteile. Europ. J. Cancer (1969), im Druck.
11. McCormick, W. J.: A collagen disease, secondary to a nutritional deficiency? Arch. Pediatr. 76 (1959), 166—171.
12. Prockop, D. J., Udenfriend, S., and Lindstedt, S.: A simple technique for measuring the specific activity of labelled hydroxyprolin in biological materials. J. Biol. Chem. 236 (1961), 1395—1398.
13. Roe, J. H., and Kuether, C. A.: Determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147 (1943), 399.
14. Roy, R. N., and Guha, B. C.: Species difference in regard for the biosynthesis of ascorbic acid. Nature 182 (1958), 319.
15. Schneider, E.: Cites Eickhorn as finding pronounced deficiency of vitamin C in cancer cases. Dtsch. med. Wschr. 79 (1951), 15.
16. Seilern-Aspang, F., and Kratochwil, K.: Induction and differentiation of an epithelial tumour in the newt (Triturus cristatus). J. Embryol. exper. Morph. 10 (1962), 337—356.
17. Seilern-Aspang, F., und Kratochwil, K.: Die Spontanheilung eines infiltrierenden und metastasierenden epithelialen Tumors von Triturus cristatus in Abhängigkeit von seinem Entstehungsort und vom jahreszeitlichen Cyclus. Arch. Geschwulstforsch. 21 (1963), 292—300.
18. Seilern-Aspang, F., und Kratochwil, K.: Experimentelle Analyse der Kontrollfaktoren, die für die Proliferation des Epithels und maligner epithelialer Tumoren von Triturus maßgeblich sind. Arch. Geschwulstforsch. 21 (1963), 113—137.
19. Seilern-Aspang, F., Wieser, W., und Weissberg, M.: Experimentelle Untersuchungen an einem epidermalen Haut-Carcinom bei Amphibien. Arch. Geschwulstforsch. 27 (1966), 201—228.
20. Seilern-Aspang, F., Mazzucco, K., und Christian, I.: Der Einfluß von Methylcholanthren auf Synthese, Vernetzung und Abbau des Kollagens der Mäusedermis und die mögliche Bedeutung dieses Abbaues für malignes Wachstum. Z. Naturforsch. 24 b (1969), im Druck.
21. Seilern-Aspang, F., Mazzucco, K., und Christian, I.: Der Einfluß von Benzol und Methylcholanthren auf die Kollagensynthese und -reifung im Granulationsgewebe. Z. Naturforsch. 24 b (1969), im Druck.
22. Seilern-Aspang, F., Edwards, W. D., Mazzucco, K., und Eder, G.: Veränderungen des Kollagengehaltes in der Schwanzhaut der Maus und das Auftreten von Carcinomen nach Transplantation in den Rücken. Europ. J. Cancer (1969), im Druck.
23. Sengel, P.: The determinism of the differentiation of the skin and the cutaneous appendages of the chick embryo. In: "The Epidermis", ed. W. Montagna and W. C. Lobitz jr., New York/London: Academic Press. (1964), p. 15—34.
24. Stuart, E. S., and Moscona, A. A.: Embryonic Morphogenesis: Role of fibrous lattice in the development of feathers and feather patterns. Science 157 (1967), 947.
25. Wessels, N. K.: Substrate and nutrient effects upon epidermal basal cells orientation and proliferation. Proc. Nat. Acad. Sci. USA 52 (1964), 252.
26. Woessner, jr. J. F.: The determination of hydroxyprolin in tissue and protein samples containing small proportions of this amino acid. Arch. Biochem. Biophys. 93 (1961), 440—447.

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Bibliography

5. Gomot, L.: Interaction ectoderme-mesoderme dans le formation des invagination uropygiennex des oiseaux. J. Embryol. exper. Morph. 6 (1958). 162-170. (Ectodermal-mesodermal interaction in formation of uropypgian invagination in birds)

10. Mazucco, K., Seilern-Aspang, F. and Chhristian, I.: Unterschiede in der Heitung des Kollagens zwischen Rucken- und Schwanzhaut von Mausen im Hinblick auf die unetrschiedliche Tumoranfalligkeit dieser Hautanteile. Europ. J. Cancer, in press. (Differences in maturation of collagen between back and tail skin of mice in relation to different tumor suspecptibility of these parts of the skin)

17. Seilern-Aspang, F., and Kratochwil, K.: Die Spontanheilung eines infiltrierenden und metastasiierenden epithelialen Tumors von Triturus cristatus in Abhangigkeit von seinem Entstehungsort und vom jahreszeitlichen Cyclus. Arch. Geschwulstforsch. 21 (1963), 292-300. (Spontaneous healing of an infiltrating and metastazing epithelial tumor of Triturus cristatus in relation to its development site and the cycle of the time of year)

18. Seilern-Aspang, F. and Kratochwil, K.: Experimentelle Analyse der Kontrollfaktoren, die fur die proliferation des Epithels und maligner epithelialer Tumoren von Triturus massgeblicher sind. Arch. Geschwulstforsch. 21 (1963), 113-137. (Experimental analysis of control factors indicative for proliferation of the epitheilum and malignant epithelial tumors of Triturus.

19. Seilern-Aspang, F., Wieser, W., and Weissberg, M.: Experimentelle Untersuchungen an einem epidermalen Haut-Carcinom bei Amphibien. Arch. Geschwulstforsch. 27 (1966), 201-228. (Experimental Investigations of an Epidermal Skin ca rcinoma in Amphibians)

20. Seilern-Aspang, F., Mazucco, K. and Christian, I.: Derm Einfluss von Methylcholanthren auf Synthese, Vernetzung und Abbau des Kollagens der Mausedermis und die mogliche Bedeutung dieses Abbaues fur Malignes Wachstum. Z. Naturforsch. 24 b (1969), in press. (The Influence of Methylcholanther on the synthesis, saturation and reduction of Collagen of mouse dermis and the possible significance of this reduction for malignant growth.)

21. Seilern-Aspang, F., Mazucco, K., and Christian, I.: Der Einfluss von Benzol und Methylcholanthren auf die Kollagensynthese und - reifung im Granulationsgewebe. Z. Naturforsch. 24 b (1969),

in press,) (The Influence of Benzol and Methylcholanther on collagen sythesis and maturation in granulation tissue).

22. Seilern-Aspang, F., Edwards, W. D., Mazucco, K., and Eder, G.:

Veranderungen des Kollagengehaltes in der Schwanzhaut der Maus und das Auftreten von Carcinomen nach Transplantation in den Rucken. Europ. J. Cancer (1969), in press.

(Changes in the collagen content of the skin of the mouse tail and the occurrence of carcinomas after transplantation into the back.)

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Arch. Geschwulstforsch. 35(1): 36-43 1970

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Aus dem Institut für Krebsforschung der Universität Wien

Beziehungen zwischen Induzierbarkeit eines Hautkarzinoms, dem Kollagengehalt der Haut und der Synthese der Askorbinsäure bei Triturus cristatus

Von G. Wirl und F. Seilern-Aspang

Mit 4 Abbildungen

(Eingang: 6. 8. 1969

Einleitung

Die Steuerung der Proliferation und Differenzierung der Epidermis während der Embryogenese (3, 5, 23) und der Regeneration — sowohl der reparativen (18) als auch der physiologischen (1) — ist bei Wirbeltieren von dem unterlagernden Bindegewebe bzw. der Dermis abhängig. In der Dermis scheint vor allem das Kollagen, wahrscheinlich durch seinen fibrillären Aufbau, von Bedeutung zu sein (7, 24, 25).

Da Kanzerisierung der Haut mit Entdifferenzierung und unkontrollierter Proliferation der Epidermiszellen einhergeht, wurden wiederholt Defekte in der Dermis als erste Phase eines malignen Wachstums der Epidermis angenommen. Ein vom Jahreszyklus abhängiges Hautkarzinom beim Molch (16, 17, 19) ergab die Möglichkeit, das Verhalten der Dermis parallel der Häufigkeit des malignen Wachstums der Epidermis zu untersuchen. War es in den Sommermonaten unmöglich, Hautkarzinome durch Wundsetzung oder durch Verabreichung von Kanzerogenen zu induzieren, so nahm im Herbst die Induzierbarkeit zu und erreichte 100 % im Januar, Februar und Anfang März. In dieser Zeit traten auch zahlreiche Spontankarzinome auf. Vorbedingung war, daß die Tiere durch Haltung bei Zimmertemperatur am Winterschlaf gehindert wurden. In den Monaten April und Mai verschwand die Karzinominduzierbarkeit völlig. Im Gegenteil, es traten Ausheilungen durch Totalverhornung der mit kleinen Hautwucherungen befallenen Tiere auf.

Histologische Untersuchungen von Hautwunden zeigten bei gleichbleibender Epithelisierung eine Abnahme des Granulationsgewebes gegen den Winter. In den Wintermonaten wurde nach Verletzung der Haut kein Granulationsgewebe gebildet. Wunden dieser Art führten zu Karzinomen: nach Epithelisierung der Wunde infiltrierten die Epithelzellen in die unterlagernden Gewebe, meist Muskulatur, von denen sie durch keine Bindegewebsstruktur getrennt waren. Malignes Wachstum entstand nur nach Zerstörung der Dermis, auch wenn die Epidermis dabei nicht verletzt wurde. Wunden der Epidermis ohne Verletzung der Dermis führten nicht zu Karzinomen. Wurde unreifes Bindegewebe aus Regenerationskegeln in eine Wunde, die kein Regenerationsgewebe mehr bilden konnte, implantiert, so regenerierte eine Dermis — es entstanden keine epidermalen Wucherungen. Bei diesen über den Winter gehaltenen Tieren erfolgte im Frühjahr wieder die Bildung von Granulationsgewebe, und es war unmöglich, durch Wundsetzung Karzinome zu induzieren. Es wurden im Gegenteil infiltrierende Hautgewächse, die noch aus dem Winter her stammen, im Frühjahr von neu auftretendem Bindegewebe abgekapselt, und die epidermalen Wucherungen verhornten völlig (16, 17).

Es zeigte sich also eine deutliche Beziehung zwischen dem Auftreten von Hautkarzinomen und der Fähigkeit, Bindegewebe aufzubauen. Dies stimmt überein mit der eingangs erwähnten Erkenntnis der Entwicklungsphysiologie, daß in der Embryogenese und Regeneration Proliferation und Differenzierung der Epidermis von Bindegewebsstrukturen gesteuert werden.

Da der Aufbau von Bindegewebe weitgehend von der Kollagensynthese abhängt, wurde der Kollagengehalt der Haut zweier Molchrassen im Jahreszyklus untersucht. Beide Rassen bekamen bei Winterhaltung Karzinome in dem oben beschriebenen Ablauf. Askorbinsäure ist für die Hydroxilierung des Prolins bei dem Aufbau eines typischen hydroxiprolinhaltigen Kollagens in vivo notwendig (6). Wundheilungsprozesse sind bei Nagern, die an Skorbut leiden, stark gestört. In Hinblick auf die zur Karzinombildung und Bindegewebsverlust führenden Bedingungen wurde daher der Askorbinsäuregehalt der Niere untersucht.

Material und Methode

Die Versuche wurden an Triturus cristatus dobrogicus aus dem Gebiet des Neusiedlersees und an Triturus cristatus carnifex aus Rumänien durchgeführt. Die Tiere wurden ganzjährig in flachen Becken zu 10 und 15 Stück bei Zimmertemperatur gehalten. Wöchentlich wurde einmal mit Tubifex gefüttert. Das Wasser wurde jeden 2. Tag gewechselt. Die Versuche wurden getrennt zwischen Männchen und Weibchen, Jungtieren und adulten Tieren durchgeführt. Da sich keine wesentlichen Unterschiede ergaben, sind diese Gruppen im nachfolgenden nicht getrennt.

1. Askorbinsäuregehalt der Niere:

Die Niere wurde deshalb für die Untersuchungen verwendet, da die Urodelen die Askorbinsäure in diesem Organ synthetisieren (14), d. h., sie sind von der durch die Nahrung aufgenommenen Askorbinsäure unabhängig. Die Nieren von 3 bis 4 Tieren wurden mittels Filterpapier von anhaftender Gewebsflüssigkeit befreit, gewogen und in Trichloressigsäure homogenisiert. Im Überstand wurde die Askorbinsäure nach der photometrischen Methode von J. H. Roe und C. A. Kuether (13) bestimmt.

Diese Methode beruht auf der Reaktion der Ketogruppe der Askorbinsäure mit 2.4 Dinitrophenylhydrazin in saurem Bereich bei 37 °C. Der Askorbinsäuregehalt wurde auf das Naßgewicht bezogen.

2. Kollagengehalt der Haut:
An beiden Seiten des Tieres, etwa in der Mitte zwischen Vorder- und Hinterextremitäten, wurden flächengleiche Hautstückchen vom Durchmesser = 0,9 cm mittels einer Rundstanze entnommen. Die anhaftende Muskulatur wurde entfernt und das Kollagen nach der Methode von D. J. Prockop, S. Udenfriend und S. Lindstedt (12) in wasserlösliche Gelatine übergeführt. Der Hydroxiprolingehalt wurde nach der photometrischen Methode von J. F. Woessner jr. (26) bestimmt.
Der Hydroxiprolingehalt der Hautstückchen wurde auf deren Fläche bezogen.

Ergebnisse

1. Der Jahresrhythmus des Kollagengehaltes der Haut

Die Abbildungen 1 und 2 zeigen den Kollagengehalt der Haut von Tieren, die im Frühjahr und im Herbst gefangen wurden. Der Kollagengehalt der Haut verläuft nach diesen Untersuchungen genau gegenteilig der Karzinomanfälligkeit. Im Frühjahr und Sommer findet sich ein Maximum an Kollagen. Zu Beginn des Winters sinkt der Kollagengehalt stark ab und erreicht seinen Tiefstand Ende Januar bis Februar und Anfang März, also zu der Zeit, wo die Induzierbarkeit von Karzinomen optimal ist und auch Spontankarzinome auftreten. Ende März bis April und Mitte Mai steigt der Kollagengehalt wieder steil an. Hier verzeichnen wir auch den Rückgang von Karzinomen und die Ausheilung kleinerer epidermaler Wucherungen.

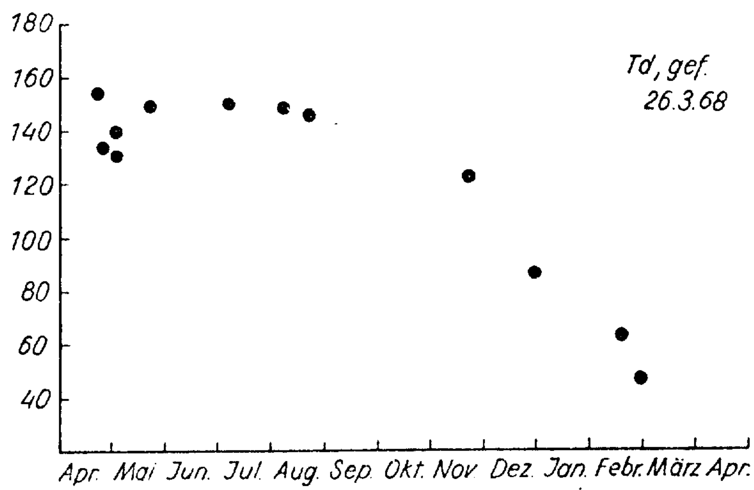

Abb. 1 Der Kollagengehalt der Haut von Triturus cristatus dobrogicus bei ganzjähriger Haltung in Zimmertemperatur.
gef. = Fangzeit
● = Durchschnitt von 4 Proben zweier Versuchstiere
μg Hypro/Hautfläche

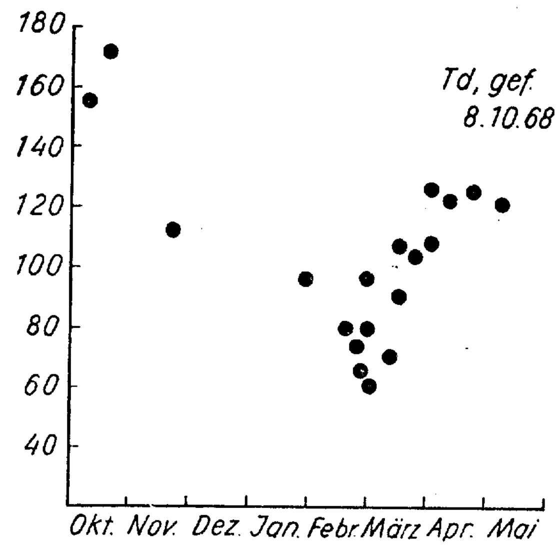

Abb. 2 Der Kollagengehalt der Haut von Triturus cristatus dobrogicus während des Winters bei Haltung in Zimmertemperatur.
gef. = Fangzeit
● = Durchschnitt von 4 Proben zweier Versuchstiere
µgH/F = µg Hypro/Hautfläche

2. Der Jahresrhythmus des Askorbinsäuregehaltes in der Molchniere

Die Abbildungen 3 und 4 zeigen den Jahreszyklus der beiden untersuchten Rassen: Bald nach Gefangenschaft erfolgt ein zunehmender Verlust an Askorbinsäure. In den Wintermonaten ist sie nicht mehr nachweisbar. Erst im Monat März steigt die Produktion derselben wieder an. Wildfänge zu verschiedenen Zeiten zeigen, daß der Verlust nicht in freier Wildbahn auftritt.

Diskussion

In unseren Untersuchungen stehen bei einer ganzjährigen Laborhaltung beider Molchrassen vier Vorgänge in einer auffälligen Beziehung. Alle vier Erscheinungen treten bei Freilandmolchen nicht auf:

1. In der zweiten Sommerhälfte fällt der Askorbinsäurespiegel. Von Oktober bis Mitte März ist keine Askorbinsäure nachweisbar. Von März an steigt er wieder bis zu einem weitgehend Normalspiegel im Mai.

2. Der wegen der Hydroxilierung des Prolins von der Askorbinsäure in vivo abhängige Kollagenaufbau (6) folgt in der Molchhaut der Synthese an Askorbinsäure. Der bei Laborhaltung rasch auftretende Rückgang derselben wird aber wegen des langsamen Turnovers des Struktur-Kollagens erst 3 bis 4 Monate später in seinem Einfluß auf das Kollagen sichtbar.

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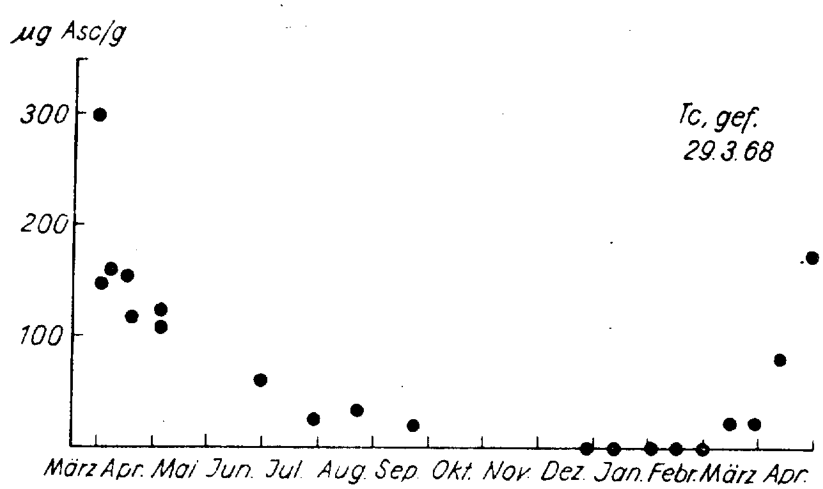

Abb. 3 Der Gehalt an Askorbinsäure der Niere von Triturus cristatus carnifex bei ganzjähriger Haltung in Zimmertemperatur.

gef. = Fangzeit

● Mittelwert der Nieren von 3 bis 4 Versuchstieren

µg Asc/g = µg Asc/g Niere

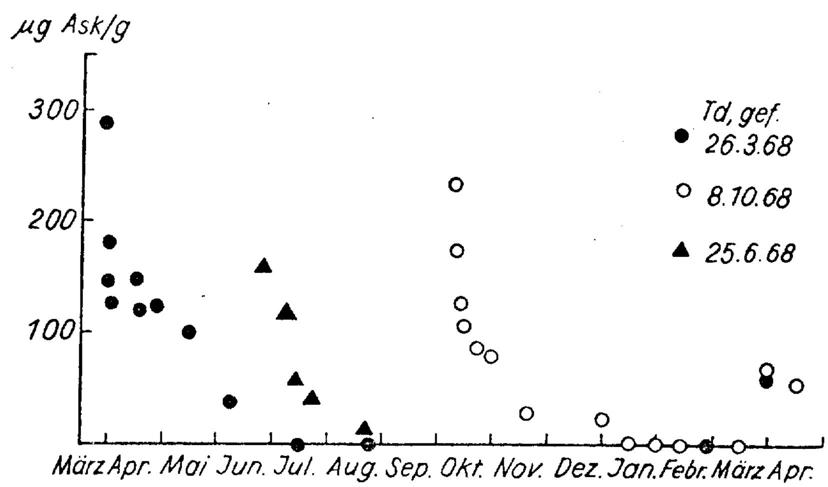

Abb. 4 Der Gehalt an Askorbinsäure der Niere von Triturus cristatus dobrogicus. Die Versuche begannen zu verschiedenen Zeitpunkten mit Wildfangen.

gef. = Fangzeit

● ○ ▲ Mittelwert der Nieren von 3 bis 4 Versuchstieren

µg Ask/g = µg Ask/g Niere

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3. Wie zu erwarten, verläuft bei Wundsetzung die Bildung von Granulationsgewebe in ihrer Quantität parallel der Kurve der Kollagensynthese. In den Zeitabschnitten, wo wenig Kollagen in der Molchhaut gefunden wird, wird auch in Hautwunden kein Granulationsgewebe gebildet.

4. Die Entstehung von Hautkarzinomen aus Hautwunden verläuft der Produktion an Askorbinsäure, dem Kollagengehalt in der Haut und der Bildung von Granulationsgewebe in Wunden antagonistisch.

Die Zunahme der Induzierbarkeit von Karzinomen durch Wundsetzung parallel der Abnahme des Kollagens in der Dermis findet sich ebenso in der Mäusehaut. Auch dort erfolgt nach Applikation eines Kanzerogens eine starke Abnahme des Kollagengehaltes (10, 20, 21). Genau wie beim Molch kann man in diesen Zonen durch Wundsetzung Karzinome verfrüht induzieren (9). Auch bei Transplantation der Schwanzhaut in den Rücken der Maus sinkt der Kollagengehalt im Transplantat fast so stark wie bei Behandlung mit Kanzerogenen (22). In solchen Implantaten kann man aus Benzol gepinselten Wunden Karzinome erzielen. Am Schwanz der Maus erfolgt dagegen durch Kanzerogene weder ein Rückgang des Kollagens noch ein malignes Wachstum der Haut. Alle diese Beispiele zeigen, daß bei verschiedenen Wirbeltieren eine Beziehung zwischen dem Entstehen epidermaler Karzinome und dem Kollagengehalt der Dermis besteht (4, 8, 10, 20, 21).

Da die Kollagensynthese vom Gehalt an Askorbinsäure abhängig ist, könnte ihre Abwesenheit einen kanzerisierenden Einfluß haben. Tatsächlich wurde häufig bei Ausbruch von Karzinomen auch ein Rückgang der Askorbinsäure beobachtet (2, 11, 15).

Betrachten wir die eingangs erwähnte Bedeutung bindegewebiger Strukturen für die Steuerung der Proliferation und der Differenzierung der Epidermis in der Normogenese, so erscheinen uns die hier auftretenden Defekte der Kollagensynthese der Dermis in ursächlicher Beziehung zu dem unkontrollierten (malignen) epidermalen Wachstum nach Wundsetzung.

Zusammenfassung

1. Bei einer ganzjährigen Laborhaltung zweier Kammolchrassen erfolgt das quantitative Auftreten eines Hautkarzinoms (spontan oder durch Wunden experimentell ausgelöst) in Form einer Kurve, deren Minimum im Frühling und Frühsommer liegt. Die Kurve steigt während des Herbstes an und erreicht ein Optimum um den Februar. Ende März erfolgt ein starker Abfall zum Minimum der Kurve.
2. Genau gegenteilig verläuft die Kurve des Askorbinsäuregehaltes in der Niere (wo die Urodelen Askorbinsäure synthetisieren), die in den Wunden auftretende Menge an Granulationsgewebe und der Gehalt an Kollagen in der Haut.
3. Da in der Embryogenese und in der Regeneration die Proliferation und Differenzierung der Epidermis von der Dermis wahrscheinlich im besonderen von der Kollagenstruktur gesteuert werden, könnte der Mangel an Askorbinsäure und damit der Abfall der Kollagensynthese wegen des Verlustes einer vollwertigen Dermisregeneration nach Wundsetzung zu einem unkontrollierten Wachstum der Epidermis führen.

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11. McCormick, W. J.: A collagen disease, secondary to a nutritional deficiency? Arch. Pediatr. 76 (1959), 166—171.
12. Prockop, D. J., Udenfriend, S., and Lindstedt, S.: A simple technique for measuring the specific activity of labelled hydroxyprolin in biological materials. J. Biol. Chem. 236 (1961), 1395—1398.
13. Roe, J. H., and Kuether, C. A.: Determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147 (1943), 399.
14. Roy, R. N., and Guha, B. C.: Species difference in regard for the biosynthesis of ascorbic acid. Nature 182 (1958), 319.
15. Schneider, E.: Cites Eickhorn as finding pronounced deficiency of vitamin C in cancer cases. Dtsch. med. Wschr. 79 (1951), 15.
16. Seilern-Aspang, F., and Kratochwil, K.: Induction and differentiation of an epithelial tumour in the newt (Triturus cristatus). J. Embryol. exper. Morph. 10 (1962), 337—356.
17. Seilern-Aspang, F., und Kratochwil, K.: Die Spontanheilung eines infiltrierenden und metastasierenden epithelialen Tumors von Triturus cristatus in Abhängigkeit von seinem Entstehungsort und vom jahreszeitlichen Cyclus. Arch. Geschwulstforsch. 21 (1963), 292—300.
18. Seilern-Aspang, F., und Kratochwil, K.: Experimentelle Analyse der Kontrollfaktoren, die für die Proliferation des Epithels und maligner epithelialer Tumoren von Triturus maßgeblich sind. Arch. Geschwulstforsch. 21 (1963), 113—137.
19. Seilern-Aspang, F., Wieser, W., und Weissberg, M.: Experimentelle Untersuchungen an einem epidermalen Haut-Carcinom bei Amphibien. Arch. Geschwulstforsch. 27 (1966), 201—228.
20. Seilern-Aspang, F., Mazzucco, K., und Christian, L.: Der Einfluß von Methylcholanthren auf Synthese, Vernetzung und Abbau des Kollagens der Mäusedermis und die mögliche Bedeutung dieses Abbaues für malignes Wachstum. Z. Naturforsch. 24 b (1969), im Druck.
21. Seilern-Aspang, F., Mazzucco, K., und Christian, L.: Der Einfluß von Benzol und Methylcholanthren auf die Kollagensynthese und -reifung im Granulationsgewebe. Z. Naturforsch. 24 b (1969), im Druck.
22. Seilern-Aspang, F., Edwards, W. D., Mazzucco, K., und Eder, G.: Veränderungen des Kollagengehaltes in der Schwanzhaut der Maus und das Auftreten von Carcinomen nach Transplantation in den Rücken. Europ. J. Cancer (1969), im Druck.
23. Sengel, P.: The determinism of the differentiation of the skin and the cutaneous appendages of the chick embryo. In: "The Epidermis", ed. W. Montagna and W. C. Lobitz jr., New York/London: Academic Press. (1964), p. 15—34.
24. Stuart, E. S., and Moscona, A. A.: Embryonic Morphogenesis: Role of fibrous lattice in the development of feathers and feather patterns. Science 157 (1967), 947.
25. Wessels, N. K.: Substrate and nutrient effects upon epidermal basal cells orientation and proliferation. Proc. Nat. Acad. Sci. USA 52 (1964), 252.
26. Woessner, jr. J. F.: The determination of hydroxyprolin in tissue and protein samples containing small proportions of this amino acid. Arch. Biochem. Biophys. 93 (1961), 440—447.

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Summary

Relation of the induction of a carcinoma of the skin to the content of collagen in the dermis and the synthesis of ascorbic acid in Triturus cristatus

1. When two races of newts are held in laboratory all the year, the quantitative appearance of the epithelial tumour (spontaneous or induced by wounding) follows a curve, whose minimum lies in spring and the first part of summer. The curve rises in autumn and the optimum is reached in February. In March there is a striking slope to the minimum of the curve.
2. However, the curve of the content of ascorbic acid in the kidneys (where ascorbic acid is synthesized in urodeles), the quantity of granulation tissue in wounds, and the content of collagen in the dermis is exactly the opposite.
3. In embryogenesis and in regeneration the proliferation and differentiation of the epidermis is controlled by the dermis, probably by the structure of collagen. Thus we would suggest that the deficiency of ascorbic acid and therefore a decrease in collagen synthesis inhibits the regeneration of the dermis in the wound. This inhibition is the cause of the uncontrolled growth of the wound epithelium.

Literatur

1. Billingham, R. E., and Silvers, W.: Studies on the conservation of epiderma specifities of skin and certain mucosas in adult mammals. J. Exper. Med. **125** (1966) 429—446.
2. Bodansky, O.: Concentration of ascorbic acid in plasma and white blood cells of patients with cancer and non-cancerous chronic disease. Cancer Res. **11** (1951), 238.
3. Cairns, J. M., and Saunders jr., J. W.: The influence of embryonic mesoderm on the regional specification of epidermal derivatives in the chick. J. Exper. Zool. **127** (1954), 221—248.
4. Gillman, T., Penn, J., Bronks, D., and Roux, M.: Possible significance of abnormal dermal collagen und of epidermal regeneration in the pathologenesis of skin cancer. Brit. J. Cancer **9** (1955), 272.
5. Gomot, L.: Interaction ectoderme — mesoderme dans le formation des invagination uropygienne des oiseaux. J. Embryol. exper. Morph. **6** (1958), 162—170.
6. Gould, B. S.: The Role od Ascorbic acid in Collagen Biosynthesis. In: "Treatise on Collagen" Vol. 2, part A. ed. B. S. Gould. London/New York: Academic Press. (1958). p. 323.
7. Grobstein, C., and Cohen, J.: Collagenase: Effect on the morphogenesis of embryonal salivary epithelium in vitro. Science **150** (1965), 626—628.
8. Hamer, D., and Marchant, J.: Collagen and other constituents in the skin of normal carcinogen-treated and castrated mice. Brit. J. Cancer **11** (1957), 445—451.
9. Mac Kenzie, I., and Rous, P.: The experimental disclosure of latent neoplastic changes in tarred skin. J. Exper. Med. **73** (1941), 391—416.
10. Mazzucco, K., Seilern-Aspang, F., und Christian, I.: Unterschiede in der Reifung des Kollagens zwischen Rücken- und Schwanzhaut von Mäusen im Hinblick auf die unterschiedliche Tumoranfälligkeit dieser Hautanteile. Europ. J. Cancer (1969), im Druck.

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J. Nutr. 20: 327: 338 1940

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THE DETERMINATION OF ASCORBIC ACID IN EVAPORATED MILK, POWDERED MILK AND POWDERED MILK PRODUCTS [1]

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(Received for publication May 18, 1940)

Within recent years evaporated and powdered milks have received some consideration as a potential source of vitamin C. This problem has apparently received little attention in America.

Schlemmer, Bleyer and Cahnmann ('32), Henriksen ('37), Palladina and Anoshkina ('37), Taniguti, Hamamoto, Hirata and Suzuki ('37), Tomoi and Tomita ('37), Henry and Kon ('38), Kon ('38) and Henry, Houston, Kon and Osborne ('39) have studied the ascorbic acid content of different milk preparations. Phospho-18-tungstic acid and various modifications of the indophenol titrimetric technique were used by them. In most cases the work on the milk preparations themselves was not verified by biological assay nor was any evidence presented as to the specificity of the reaction involved in the analysis.

Further study of the photoelectric method suggested by Woessner, Elvehjem and Schuette ('39) has disclosed certain improvements especially valuable when determining dehydroascorbic acid. We wish therefore to describe the improved method as applied to evaporated milk, powdered milk, and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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powdered milk products and to cite experimental evidence that the method is indeed specific for ascorbic acid.

EXPERIMENTAL PART

The samples of evaporated milk were prepared by diluting one part of the evaporated milk with one part of copper-free water. Samples of powdered milks and powdered milk products were prepared by dissolving 145 gm. of the powder in 1 liter of copper-free water. Heating to 50°C. was necessary in order to bring the roller-dried products into solution. All powders were stirred into the water with an ordinary silver-plated spoon.

The samples were analyzed for both the reduced and reversibly oxidized ascorbic acid. The reduced ascorbic acid was determined according to the procedure already described by the authors ('39). However a very slow sliding of the galvanometer is always observed with evaporated milk. Thus it is necessary to take the galvanometer reading not later than 15 seconds after the indophenol has been added to the colorimeter tube if the true reduced ascorbic acid is to be determined accurately.

The procedure for the determination of the dehydroascorbic acid has been modified as follows: After the addition of a few drops of dibutyl phthalate to the milk in order to prevent foaming, wet hydrogen sulfide is bubbled through the milk for exactly 20 minutes. Then as rapidly as manipulation will permit, 25 ml. of the hydrogen-sulfide-saturated milk are added to 75 ml. of modified Willberg reagent and the whole shaken well to break the resulting curd into small particles. The hydrogen sulfide is removed immediately by passing a vigorous stream of wet oxygen-free nitrogen through the suspension. Our experience has shown that this operation takes no longer than 20 minutes for 50 ml. of the curd-reagent mixture. After the curd is removed by filtration, 5 ml. of the filtrate are measured into one of the colorimeter tubes. We have never experienced difficulty in obtaining a crystal-clear serum at this point. Then with the simultaneous start of a stop watch

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10 ml. of the dye-acetate solution are added to the tube by means of a rapidly delivering pipette and the galvanometer readings at 15 and 30 seconds are recorded. The galvanometer reading corresponding to zero seconds, for practical purposes, can be considered equal to the difference of the galvanometer readings at 15 and 30 seconds subtracted from the galvanometer reading at 15 seconds.

The calculations and other details of this modified procedure are identical with those described in the original paper. This modified procedure is also recommended for use with whole milks, raw and pasteurized, as it gives more reliable results especially when determining dehydroascorbic acid.

DISCUSSION

Experiments conducted with dehydroascorbic acid solutions in 10% aqueous sodium chloride prepared either by iodine oxidation or use of ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35) showed that at hydrogen ion concentrations comparable to those of fresh milk and milk preparations recovery of dehydroascorbic acid was quantitatively complete when hydrogen sulfide was introduced for 20 minutes or longer. It was not possible to obtain 100% recovery at a pH 2 or lower even after 8 hours of hydrogen sulfide treatment. This is in accordance with the experiences of Bessey ('38) but not in agreement with the observation of Kon and Watson ('36).

Like de Haas and Meulemans ('36), we have observed that hydrogen sulfide treatment of milk reduces other substances besides dehydroascorbic acid. Since these reduced substances react with the indophenol at a slower rate than ascorbic acid at the final pH used in the colorimeter tube, the interference can be eliminated by extrapolation to zero time. However if the hydrogen sulfide treatment is extended much beyond 20 minutes, it becomes increasingly difficult to extrapolate with acceptable accuracy.

Heated lactose solutions (pH 6) introduce no error. However when heated lactose solutions were treated with hydrogen

sulfide under the conditions of the experiment a very slow movement of the galvanometer was observed. The interference was not serious and could be eliminated by extrapolation to zero time.

Redissolved washed casein did not contribute to the formation of interfering substances upon hydrogen sulfide treatment. It was found that at the pH of milk riboflavin offered no interference as it did with the original method at the higher hydrogen ion concentrations. Hydrogen sulfide treatment of lactoglobulin solutions prepared from evaporated milks produced a marked interference very similar to that observed under the conditions of the procedure suggested. This interference also could be eliminated by extrapolation to zero time. The lactalbumin fraction of milk also indicated formation of interfering substances on treatment with hydrogen sulfide but to a much less marked degree. This interference was likewise successfully eliminated by extrapolation.

The fact that these effects were more marked with the more drastically heated milk products than with the raw or pasteurized milks, seems to indicate that reducible soluble proteins or protein fractions have been formed during the processing. It is very likely also that some of these reducible substances are sulfhydryl compounds. Josephson and Doan ('39) reported that sulfhydryl compounds are formed in milk if it is heated to a sufficiently high temperature. Their observation that these compounds are readily oxidized with copper is in agreement with the observations made in this laboratory. They suggest that the lactalbumin of the milk is the most likely ingredient responsible for this condition and state that the protein of the fat globule adsorption membrane may also be involved.

Since qualitative examination of a white flocculent precipitate obtained from a strongly ammoniacal evaporated milk serum gave a positive test for tin and a very faint test for iron, the reaction of these metals in the procedure was studied. Stannic and stannous tin solutions did not accelerate the oxidation of ascorbic acid under semi-anaerobic conditions.

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Stannic tin itself does not interfere although when treated with hydrogen sulfide it contributed to the formation of stannous tin which partially remains in solution under the conditions of the method. At the final pH in the colorimeter tube (4.1) the stannous tin reacts at a slower rate than the ascorbic acid. Hence no error is introduced if the readings are extrapolated to zero time.

The case of iron is more serious. Ferric iron does not react with 2,6-dichlorophenolindophenol but the hydrogen sulfide treatment always produces some ferrous iron which remains in solution. The ferrous iron reacts instantaneously with the indophenol at the final pH of the solution in the colorimeter tube. Since milk picks up, at most, an insignificant quantity of iron from the processing which it undergoes, the error introduced from this source under ordinary conditions is nil. A sample of evaporated milk, by analysis found to contain a total of 15 mg. of ascorbic acid per liter, was treated for 15 minutes with the ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35). Analysis then showed no reduced ascorbic acid present but treatment with hydrogen sulfide for 20 minutes indicated 15 mg. of dehydroascorbic acid per liter. If the oxidase-treated milk was heated to 100°C. for 10 minutes, the analysis showed no ascorbic acid. However if either copper, hydrogen peroxide or intense irradiation was used to destroy completely the ascorbic acid present, analyses would always indicate reducing activity following hydrogen sulfide treatment. Since no reducing activity was ever obtained after hydrogen sulfide treatment, when use was made of ascorbic acid oxidase followed by heat, it is deemed that a non-specific oxidizing procedure, besides causing the irreversible destruction of ascorbic acid, will also cause the formation of substances which, when reduced with hydrogen sulfide, are capable of instantaneous reaction with indophenol at pH 4.1. If iron had been present in sufficient quantity to interfere, the oxidase-treated heated sample would have given a positive value for dehydroascorbic acid. Furthermore, tests on the serum of the milk before and after hydrogen sulfide treatment

failed to give a positive reaction for iron. The value of the ascorbic acid oxidase in deciding whether or not all reducing activity is due to ascorbic acid is only valid if interference due to stannous tin and reduced proteins can be eliminated, for we have observed that ferrous iron and stannous tin are oxidized by ascorbic acid oxidase. Since copper also catalyzes these reactions, this may be evidence in support of the essential constituent of the enzyme.

Further assurance that the method is specific for ascorbic acid was given by biological assay of samples of evaporated milk which showed wide variance in their ascorbic acid content. The same samples showed similar potency by biological and chemical assay.

Results of the determinations by method presented. The results compiled in tables 1 and 2 were secured in the normal process of testing the method under working conditions. Since it was not possible to obtain information as to the age of the samples, the time of the year they were prepared or other pertinent data, they are included here to give an indication of the ascorbic acid content of powdered and evaporated milks as purchased throughout bakery, grocery, and drug stores in a city of moderate size. A few general conclusions may also be drawn from the data.

It is apparent from tables 1 and 2 that the ascorbic acid content of powdered milk is roughly equal to that of evaporated milks. The powdered milk products, however (items 8 and 9 in table 1 for example), are for the most part significantly inferior to powdered milks and the average evaporated milk. It appeared that neither the spray process, the roller process, nor even the mode of storage, is the significant factor which determines the ascorbic acid content of the powder. It is possible that, unless special precautions are used, a greater quantity of heat-labile dehydroascorbic acid is formed the more the powdered milk and powdered milk products are handled or modified. Thus, when the dehydration is brought about by heat, the destruction of the antiscorbutic potency is in direct proportion to the quantity of dehydroascorbic acid

TABLE 1

Ascorbic acid content of powdered milks and powdered milk products

MATERIAL TESTED	ASCORBIC ACID PER LITER[1]			
	Reduced	Oxidized	Total	Per kg. of powder
	mg.	mg.	mg.	mg.
1. Open barrel, skim, spray	9.0	2.4	11.5	78.8
2. Open barrel, skim, spray	1.3	1.2	2.6	17.9
3. Closed tin, skim, roller	7.2	1.8	9.1	62.7
4. Open barrel, whole, roller	9.6	4.4	14.0	97.1
5. Vacuum, whole, spray	9.4	1.2	10.6	68.6
6. Spray dried, modified by addition of milk fat and sugar. Vacuum	7.9	0.4	8.3	57.9
7. Superior quality milk, part of fat removed, fortified by rice extract; irradiated and dried by roller process, vacuum	13.4	1.2	14.6	100.8
8. Protein milk, high in protein, lactic acid and low in lactose, pH 4.8, vacuum	2.6	4.3	6.9	48.0
9. Soluble casein, lactalbumin, lactose, milk salts, dextrins, maltose, extracts of yeast and wheat embryo. Vacuum	1.8	0.0	1.8	12.6

[1] Powder was dissolved to give a milk containing 12.5% of total solids.

TABLE 2

Ascorbic acid content of commercial evaporated milk

PRODUCT TESTED		ASCORBIC ACID PER LITER[1]		
		Reduced	Oxidized	Total
		mg.	mg.	mg.
Brand 1		3.0	0.8	3.8
Brand 2		3.9	1.5	5.4
Brand 1		4.6	2.4	7.0
Brand 3		4.9	4.7	9.6
Brand 4		8.0	1.6	9.6
Brand 5		11.2	1.5	12.7
Brand 6		11.8	1.9	13.7
Brand 7		10.0	4.1	14.1
Brand 8	Irradiated	19.5	2.8	22.3
Brand 8	Irradiated	20.1	0.3	20.4
Brand 9	Zuker Process	13.7	5.7	19.5
Brand 10	Irradiated	7.8	0.1	7.9
Brand 11	Irradiated	1.7	5.7	7.4
Brand 10	Irradiated	6.5	0.2	6.7
Evaporated goats' milk		14.8	0.0	14.8

[1] As milk comes from can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

present. Consequently it appears that powdered milks are potentially a significant source of antiscorbutic factor if they are prepared from a milk which is rich in reduced ascorbic acid at the time of manufacture.

We have observed that milk can be aerated at 100°C. for 5 minutes with small loss of ascorbic acid. Consequently it is not surprising to find that a milk powder dissolved at 20°C. or at 50°C. will yield milks which show no detectable difference in their ascorbic acid content if the milk prepared at the higher temperature is cooled soon after its preparation.

The data of table 2 reveal that the ascorbic acid content of irradiated evaporated milks is not significantly different from that of the non-irradiated. This is understandable in view of the observation made by Scheer ('39) that only a slight reduction in ascorbic acid occurs when milk is irradiated with the intent of fortifying it with vitamin D and that this reduction is without practical significance.

The Evaporated Milk Association cooperated with this investigation in the collection of twenty-one samples of evaporated milk manufactured during the first week of April, 1940, in various plants throughout this country. The analyses of these samples are given in table 3. The sterilization temperature was 242° F. for 14 minutes and the forewarming or preheating temperatures varied from 204° F. to 212° F. Analyses were completed 12 to 16 days after the samples were manufactured.

The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk. This is proved by the wide variance in ascorbic acid shown by milks manufactured in equipment consisting of various combinations of copper, tinned copper and stainless steel. Evaporated milks manufactured almost entirely in copper equipment may contain more ascorbic acid than some evaporated milks manufactured almost entirely in stainless steel equipment.

The survival of any ascorbic acid at all while the milk is being subjected to evaporation in a copper pan may be ex-

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plained by the presence of sulfides which are formed when the milk is heated, as well as a scarcity of oxygen. The sulfides possibly combine with any trace of copper which may dissolve and also act as reducing agents during the process. The latter point is not entirely a speculation in the light of observations by Sharp, Trout and Guthrie ('36) and Josephson and Doan ('39) who showed that ascorbic acid is protected to a considerable degree when the milk is heated to sufficiently high temperatures even when small amounts of copper are present. Josephson and Doan ('39) postulate that this action

TABLE 3

Ascorbic acid content of evaporated milks[1]

LOCATION OF PLANT	MATERIAL OF CONSTRUCTION[2]			ASCORBIC ACID PER LITER[3]		
	Pre-heater	"Hot wells"	Evaporating pan	Reduced	Oxidized	Total
				mg.	*mg.*	*mg.*
Colorado	none	Sn-Cu	Cu	24.0	3.6	27.6
Idaho	none	Ni	Cu	13.8	2.0	15.8
Illinois	Cu	S.S.	Cu	13.3	2.5	15.8
Indiana	S.S.	S.S., Cu	S.S.	15.8	0.0	15.8
Iowa	S.S.	Sn-Cu	Cu	12.0	0.0	12.0
Kentucky	Sn-Cu	Ni	Cu	7.2	0.8	8.0
Maryland	S.S.	Cu	S.S.	5.0	1.2	6.2
Michigan	S.S.	S.S., Cu	S.S.	6.2	1.8	8.0
Minnesota	Cu	Sn-Cu	Sn-Cu, S.S.	22.4	1.6	24.0
Mississippi	none	Ni	Cu	7.2	2.8	10.0
Missouri	S.S.	S.S. or Cu-Ni alloy	S.S.	11.6	2.2	13.8
Ohio	S.S.	Sn-Cu	Cu	11.6	2.2	13.8
Oregon	S.S.	Sn-Cu, S.S.	Cu	10.0	3.8	13.8
Tennessee	S.S.	Ni	Cu	5.0	3.0	8.0
Texas	Sn-Cu	Sn-Cu	Cu	4.6	2.6	7.2
Utah	none	Ni	Cu	20.8	0.0	20.8
Virginia	S.S.	S.S.	Cu	6.7	3.3	10.0
Washington	S.S.	Sn-Cu	Cu	4.0	0.0	4.0
Wisconsin	S.S.	S.S.	Cu	17.6	1.6	19.2
Wisconsin	none	Cu	Cu	14.8	1.0	15.8
Wisconsin	Cu	glass	Cu	7.7	0.8	8.5
			Average	11.5	1.7	13.2

[1] Supplied by the Evaporated Milk Association.
[2] Sn-Cu, tinned copper; S.S., stainless steel.
[3] As milk comes from the can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

is due to the formation of sulfhydryl compounds in the milk at the elevated temperatures.

We have observed that its dehydroascorbic acid is completely destroyed when whole milk, containing about equal quantities of ascorbic and dehydroascorbic acids, is heated for only 10 minutes at 100°C. Thus it is understandable that the dehydroascorbic acid content of evaporated milk is insignificant and for practical purposes its determination could be omitted. The very small quantity of dehydroascorbic acid found indicates that this acid is completely destroyed during the evaporation and sterilization of the milk. Apparently sulfhydryl compounds or sulfides cannot inhibit the thermal rupture of dehydroascorbic acid. Consequently the actual quantity of ascorbic acid which survives during the processing is largely dependent on the quantity of the acid in the reduced form in the milk as it is received before concentration.

In attempting to prevent ascorbic acid losses it is apparent, from table 3, that either the omission or inclusion of a preheating step in the process of manufacture is not important. Likewise neither the quality nor the quantity of the stabilizing salts used exerts any influence here. Moreover the ascorbic acid content cannot be correlated with geographical site of production of the evaporated milk.

If the values cited for commercial evaporated milks in tables 2 and 3 are divided by two in order to express them on the basis of whole milk, we find that the average American evaporated milk supplies 25% of the quantity of ascorbic acid found in freshly drawn cow's milk. It should be emphasized, also, that this observation is based upon the analyses of evaporated milk samples which were fresh, that is, stored for no longer than 2 weeks. That a different picture may be obtained after the evaporated milks have been stored for a longer period, is quite probable inasmuch as Henry, Houston, Kon and Osborne ('39) observed that ascorbic acid is lost slowly but steadily under such conditions. We have a range, however, of 1.9 to 13.8 mg. of ascorbic acid per liter. This range indicates that evaporated milk is potentially a significant source of ascorbic acid. Raw milk contains on an average 22.6 mg. of ascorbic acid per liter. This demonstrates that in certain of the samples of evaporated milk only about a 50% loss of ascorbic potency has occurred. The maximum value of 13.8 mg. per liter is not much lower than the average value of 17.3 mg. per liter, which we have reported for commercial raw milk, nor is it significantly different from the average value of 12.6 mg. per liter which we have reported for commercial pasteurized milk.

SUMMARY

The improvements of the method previously suggested by the authors make the method less time-consuming and the determination of dehydroascorbic acid more reliable. Among the substances found to make the use of the photoelectric colorimeter essential are heated lactose, stannous tin and certain of the water soluble proteins or protein fractions. The reliability of the photoelectric colorimeter is limited only by iron which fortunately is not present in significant quantities in milk or milk products.

The analyses of forty-one evaporated milks and nine powdered milks are given. The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk.

LITERATURE CITED

Bessey, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of reducing substances. J. Biol. Chem., vol. 126, p. 771.

de Haas, J. H., and O. Meulemans 1936 The ascorbic acid content of human milk in Batavia (trans. title). Geneeskund. Tijdschr. Nederland-Indie, vol. 76, p. 2277.

Henriksen, P. K. 1937 Vitamins and milk. Ingeniøren, vol. 46, p. 61; 1938, Chem. Zentr., vol. 109, I, p. 1025.

Henry, K. M., J. Houston, S. K. Kon and L. W. Osborne 1939 The effect of commercial drying and evaporation on the nutritive properties of milk. J. Dairy Res., vol. 10, p. 272.

Henry, K. M., and S. K. Kon 1938 The effect of commercial sterilization on the vitamin C content of milk. J. Dairy Res., vol. 9, p. 185.

Josephson, D. V., and F. J. Doan 1939 Cooked flavor in milk. Milk Dealer, vol. 29, no. 2, p. 35.

Kon, S. K. 1938 The effect of commercial sterilization on nutritive values of milk. VII. Conclusions. J. Dairy Res., vol. 9, p. 207.

Kon, S. K., and M. B. Watson 1936 The effect of light on the vitamin C of milk. Biochem. J., vol. 30, p. 2273.

Palladina, O. K., and A. A. Anoshkina 1937 The vitamin C content of milk and its synthesis during lactic fermentation. Microbiology (U.S.S.R.), vol. 6, p. 787; 1938, Chem. Zentr., vol. 109, I, p. 459.

Sharp, P. F., G. M. Trout and E. S. Guthrie 1936 Vitamin C, copper and the oxidized flavor of milk. Tenth Ann. Report. N. Y. State Assoc. of Dairy and Milk Inspectors, p. 153.

Scheer, K 1939 Schädigt die Ultraviolettbestrahlung der Milch ihren Vitamin-C-Gehalt? Münch. Med. Wochschr., vol. 86, p. 603.

Schlemmer, F., B. Bleyer and H. Cahnmann 1932 Studien über Biochemishe Aktivatoren der Milch. Biochem. Z., vol. 254, p. 187.

Taniguti, T., E. Hamamoto, Y. Hirata and K. Suzuki 1937 Kritik über die Vitamin-C-bestimmung nach indophenoltitration. Orient. J. Diseases Infants, vol. 21, p. 1.

Tauber, H., I. S. Kleiner and D. Mishkind 1935 Ascorbic acid (vitamin C) oxidase. J. Biol. Chem., vol. 110, p. 211.

Tomoi, T., and S. Tomita 1937 Vitamin C contents of different milk preparations. Sei-i-kai Med. J., vol. 56, p. 469; C. A., vol. 31, p. 8725[a].

Woessner, W. W., C. A. Elvehjem and H. A. Schuette 1939 The determination of ascorbic acid in commercial milks. J. Nutrition, vol. 18, p. 619.

J. Nutr. 18: 619-626 1939

THE DETERMINATION OF ASCORBIC ACID IN COMMERCIAL MILKS [1]

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(Received for publication August 7, 1939)

Milk as a source of vitamin C has attracted considerable attention recently. Holmes, Tripp, Woelffer and Satterfield ('39) have reviewed available data and have studied the breed and seasonal variations in the ascorbic acid content of certified milk from Guernseys and Holsteins. Our interest lies in the ascorbic acid content of regular commercial samples of milk as related to the quantity present in the milk at the time of milking.

EXPERIMENTAL PART

The procedure described by Mindlin and Butler ('38) which involves the use of the Evelyn photoelectric colorimeter ('36) for the determination of ascorbic acid in blood plasma was modified so as to be applicable to milk.

The oxidation of ascorbic acid other than by 2, 6,-dichlorophenolindophenol even in the presence of added copper is prevented by the use of a solution developed by Willberg ('38) which consists of sodium chloride and oxalic acid. It was modified by us to contain also metaphosphoric acid with the result that a clearer serum is obtained and the formation of a precipitate in the colorimeter tube is prevented when the dye-acetate solution is added. Loss of reducing activity is prevented in the filtrate for 16 hours if exposure to direct sunlight or strong illumination is avoided.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The volume of milk serum and quantity of dye-acetate solution are so controlled that the usual concentration of vitamin present reacts almost instantaneously. Under these same conditions the interfering substances formed in the milk after hydrogen sulfide treatment react at a slower rate, thus enabling their interference to be largely eliminated by extrapolation to zero time.

Among the interfering substances which are formed upon treatment of the milk with hydrogen sulfide, no doubt one of them is reduced riboflavin. Experimentation with ascorbic acid-riboflavin solutions under similar conditions indicates this to be the case. Apparently more riboflavin is reduced, the longer the milk serum remains in contact with the reductant, 2 hours being the average time when the effect first becomes noticeable. Fortunately, the reduced riboflavin and other substances react more slowly than ascorbic acid under the conditions used and therefore this interference is largely eliminated by extrapolation. It is to be noted that a titrimetric procedure employed on a milk serum which has been treated with hydrogen sulfide long enough to reduce the dehydroascorbic acid will invariably give results which are too high and the error involved will be dependent upon two factors—the time during which the serum has been left in contact with hydrogen sulfide and the time actually employed for the completion of the titration. Hence the use of the colorimeter or some similar instrument is essential for the determination of the dehydroascorbic acid if the effect of other reductants formed under the conditions needed to reduce the dehydroascorbic acid is to be largely eliminated.

The apparatus is identical with that used by Mindlin and Butler ('38) as is the 2, 6,-dichlorophenolindophenol, sodium acetate and indophenol acetate solutions. The potassium oxalate, cyanide and metaphosphoric acid solutions are, however, replaced by a modified Willberg ('38) reagent which is prepared accurately in order to insure the proper pH in the colorimeter tube, by dissolving in 100 ml. of glass-distilled water 0.6 gm. $H_2C_2O_4 \cdot 2H_2O$, 4.8 gm. NaCl C.P. and 6.5 gm.

HPO_3 (reagent, grade, sticks). This reagent is stable for 1 week at 15 to 20°C. and remains at constant pH.

Determination of ascorbic acid. In the absence of strong light 25 ml. of milk are pipetted into a 125 ml. Erlenmeyer flask containing 75 ml. of modified Willberg reagent. The protein precipitate is removed by filtering through paper of quality similar to Whatman 42.

Five milliliters of the filtrate are measured into a colorimeter tube and 10 ml. of the indophenol-acetate solution are added. The contents are stirred and read immediately.

Since it is not always possible to obtain a filtrate that is crystal-clear, it is recommended that the suggestion of Bessey ('38) be used; that is, a small crystal of ascorbic acid is added after the original reading has been made whereupon the correction due to the prevalent turbidity is determined. Thus the true reading is equal to the original reading plus 100 minus the reading after the crystal of ascorbic acid has been added.

Determination of dehydroascorbic acid. After the addition of a few drops of dibutyl phthalate to prevent foaming, hydrogen sulfide is bubbled through the remainder of the filtrate for 5 minutes. The tightly stoppered flask is allowed to stand for 8 hours in the dark at room temperature. The hydrogen sulfide is removed by passing a vigorous stream of wet oxygen-free nitrogen through the filtrate for 30 to 45 minutes. In order to remove the elemental sulfur and the dibutyl phthalate, the contents of the flask are filtered through paper of quality similar to Whatman 42.

Five milliliters of the filtrate are measured into one of the colorimeter tubes. With a simultaneous start of a stop watch, 10 ml. of the dye-acetate solution are blown into the tube and the galvanometer readings at 15, 30, 45, 60, 90 and 120 seconds are recorded. After the one hundred and twentieth second a few crystals of ascorbic acid are added and all these readings are corrected in the manner already described for the slight amount of turbidity that invariably seems to be present. The logarithms of the difference between

100 minus the galvanometer readings plotted against time and extrapolated to zero time will give the most accurate galvanometer reading due to the ascorbic acid plus the dehydroascorbic acid.

We have found that under the conditions described K has a value of 0.166 ± 0.003 for different lots of indophenol (practical). It is to be noted that when determining this value it is essential to have the same final pH in the colorimeter tubes as that obtained when a sample of milk is substituted for the standard ascorbic acid solution. This may readily be accomplished if the standard ascorbic acid solution employed is made by diluting three parts of modified Willberg reagent with one part of water. Other details and calculations have already been described by Mindlin and Butler ('38) and Bessey ('38). Typical recovery experiments have demonstrated a per cent recovery from 94 to 98.

DISCUSSION

Knight, Dutcher and Guerrant ('39) have shown conclusively that there is no adsorption of ascorbic acid on the precipitated proteins and are of the opinion that as the milk leaves the udder of the cow, its ascorbic acid is all in the reduced form. We have confirmed the latter point and have noted that, if the milk is immediately pipetted into the modified Willberg reagent directly after the milking machine is detached from the udder of the cow, treatment with hydrogen sulfide is unnecessary as no significant oxidation occurred during the milking operation and that the reagent prevented any further oxidation until the analysis could be completed.

Results of determinations by method presented. Recorded in table 1 are the analyses of milks of Holstein, Brown Swiss, Jersey and Guernsey cows in the regular University of Wisconsin herd. These analyses were made in January when the animals were on winter feed. The samples were secured immediately after the machine was detached from the udder and pipetted directly into the Willberg reagent. The samples were collected on 3 consecutive days during the afternoon

TABLE 1

D[illegible]tions in the ascorbic acid content of milk from individual cows taken directly after milking

HOLSTEIN (A)[1]	ASCORBIC ACID PER LITER 1st day	2nd day	3rd day	PM MILKING POUNDS MILK
	mg.	*mg.*	*mg.*	
3	14.6	15.3	14.7	20
7	21.2	21.3	21.5	14
9	26.2	25.0	25.7	15
10	18.5	....	18.9	9
11	25.6	23.3	29.2	24
12	17.0	18.4	16.7	58
18	23.2	18.6	18.3	18
29	21.3	21.7	19.9	6
Average	20.9	20.5	20.6	
HOLSTEIN (B)[1]				
52	18.8	19.5	19.1	5
58	28.6	28.4	23.6	7
63	23.9	23.0	23.6	10
64	17.6	18.6	18.6	8
65	21.7	23.0	21.2	14
67	23.0	25.1	25.1	21
83	22.1	17.1	19.1	8
84	22.0	21.3	22.4	8
91	19.9	20.0	20.5	17
Average	21.7	21.6	21.7	
BROWN SWISS				
805	23.0	22.0	23.6	12
811	28.6	26.6	28.6	23
815	20.0	18.6	19.1	19
814	27.5	27.5	27.4	11
822	23.6	23.3	23.2	7
827	26.4	26.8	25.6	6
834	28.0	29.3	28.6	8
839	25.8	26.2	25.1	11
840	....	20.0	29.2	9
Average	25.3	24.4	25.6	
JERSEY				
608	21.0	20.8	20.8	7
621	23.6	24.4	20.0	6
623	31.2	29.6	29.0	14
639	15.4	16.7	17.6	7
640	31.8	29.4	27.8	10
656	18.8	22.4	21.6	8
660	22.7	24.8	22.9	10
661	23.7	20.2	25.1	14
Average	23.5	23.5	23.1	
GUERNSEY				
404	22.5	21.6	22.0	8
412	23.3	22.0	23.6	20
417	22.7	22.7	24.2	13
421	21.7	24.0	23.3	17
424	19.2	19.6	20.0	13
427	27.5	29.6	29.6	9
428	24.4	23.9	25.2	11
432	24.4	24.2	24.8	10
437	18.6	16.8	17.7	9
438	21.7	22.7	23.3	13
441	22.5	24.8	24.0	13
447	20.5	22.1	21.0	7
450	18.8	20.4	21.2	10
455	18.0	18.0	18.0	15
457	20.8	20.0	22.7	16
415	26.6	27.2	27.5	11
Average	22.2	22.4	23.0	

[1] (A) group on winter feed the year round. (B) group had summer pasture available yearly.

milking. It will be noted that the daily values for the ascorbic acid are very constant for any individual animal, that the quantity of ascorbic acid produced per liter varies significantly with each animal and that a high producer does not necessarily give less ascorbic acid per liter than a low producer.

It is further observed that milk from the Brown Swiss herd contained the most ascorbic acid per liter followed by Jersey, Guernsey and Holstein, respectively, and that the magnitude of the figures for the Guernsey and Holsteins are in close agreement with those reported by Holmes, Tripp, Woelffer and Satterfield ('39).

Samples of raw, certified, certified Guernsey and certified vitamin D milks were collected at the different dairies throughout the city of Madison. These milks (table 2) on the average are only a little below the fresh milks as recorded in table 1, indicating that commercial raw and certified milks as delivered to the consumer lose only a small amount of their antiscorbutic potency. Likewise, samples of commercial pasteurized milks were collected and analyzed. On an average they contained only about one-half as much ascorbic acid as fresh raw milks and significantly less ascorbic acid than the commercial unpasteurized milks. These results are recorded in table 3.

In both the pasteurized and unpasteurized milks the amount of dehydroascorbic acid is less than the ascorbic acid. The average percentage of dehydroascorbic acid present in the pasteurized milks makes up less of the total than in the case of unpasteurized milks. This is probably indicative of the tendency of pasteurization to form more dehydroascorbic acid which, once formed, is more readily destroyed.

Commercial pasteurized vitamin D milks in which the potency has been increased by incorporating vitamin D concentrates directly into the milk by homogenization fall below the average value of ordinary pasteurized milks in respect to the ascorbic acid content (table 4). On the other hand, if the vitamin D content has been increased by feeding the animal irradiated yeast, the antiscorbutic property falls on a par with

TABLE 2

Ascorbic acid content of commercial raw milks

COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
	mg	*mg*	*mg*
Grade A raw	7.9	8.8	16.7
Certified dairy 1	13.1	6.3	19.4
Certified dairy 2	13.7	4.8	18.5
Certified dairy 3	15.1	4.0	19.1
Certified dairy 4	12.8	8.8	21.6
Grade A Guernsey raw	7.1	13.0	20.1
Certified Guernsey dairy 1	8.2	5.0	13.2
Certified Guernsey dairy 2	16.2	2.5	18.7
Certified vitamin D from C.L.O. (1)	7.9	6.0	13.9
Certified vitamin D from C.L.O. (2)	7.9	4.2	12.1
Average	10.9	6.3	17.3

TABLE 3

Ascorbic acid content of commercial pasteurized milks

PASTEURIZED COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
	mg.	*mg.*	*mg.*
Guernsey dairy 1	3.8	1.8	5.6
Guernsey dairy 2	14.0	2.6	16.6
Guernsey dairy 3	7.8	8.8	16.6
Milk dairy 1	5.7	3.8	9.5
Milk dairy 2	14.0	1.1	15.1
Milk dairy 3	10.1	3.4	13.4
Milk dairy 4	12.4	5.3	17.7
Milk dairy 5	6.2	3.2	9.4
Grade A dairy 1	1.9	5.5	7.4
Grade A dairy 2	14.0	1.1	15.1
Average	8.9	3.5	12.6

TABLE 4

Ascorbic acid content of commercial pasteurized and modified milks

PASTEURIZED COMMERCIAL MILK	ASCORBIC ACID PER LITER		
	Reduced	Oxidized	Total
Vitamin D grade A (irradiated ergosterol)	3.7	3.7	7.4
Vitamin D (irradiated ergosterol added)	4.1	5.7	9.8
Vitamin D (feeding irradiated yeast)	6.9	6.8	13.7
Homogenized vitamin D (feeding irradiated yeast)	1.7	9.4	11.1
Mineral modified	2.7	5.4	8.1

the average value for commercial pasteurized milks. This would indicate that homogenization tends to destroy ascorbic acid.

Mineral modified milk is low in ascorbic acid and this is doubtless to be expected considering the treatment to which the milk must be subjected.

SUMMARY

A convenient method for the determination of ascorbic and dehydroascorbic acid in raw and commercially pasteurized milks has been described. The photoelectric colorimeter makes possible the elimination of many of the interfering substances which are formed when the milk is treated with hydrogen sulfide. Among these substances is reduced riboflavin.

The influence of breed as well as milk production has been observed as affecting the quantity of ascorbic acid produced. No daily variation in the milk of individual cows was observed.

It was found that commercial raw milks contained an antiscorbutic potency which was only slightly less than fresh raw milks and that pasteurized milks on the average contained only one-half the latter potency. Mineral modification and homogenization apparently have a destructive effect on ascorbic acid.

LITERATURE CITED

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored substances in the presence of other reducing substances. J. Biol. Chem., vol. 126, p. 771.

EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., vol. 115, p. 163.

HOLMES, A. D., F. TRIPP, E. A. WOELFFER AND G. H. SATTERFIELD 1939 A study of breed and seasonal variations in the ascorbic acid content of certified milk from Guernseys and Holsteins. J. Nutrition, vol. 17, p. 187.

KNIGHT, C. A., R. A. DUTCHER AND N. B. GUERRANT 1939 The quantitative determination of vitamin C in milk. Science, vol. 89, p. 183.

MINDLIN, R. L., AND A. M. BUTLER 1938 The determination of ascorbic acid in plasma, a macro and micromethod. J. Biol. Chem., vol. 122, p. 673.

WILLBERG, B. 1938 Die Bestimmung von Vitamin C in Milch. Z. Unters. Lebensm., vol. 76, p. 128.

Biochem. J. 37: 695-702 1943

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Apparent Vitamin C in Foods

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(*Received 12 August* 1943)

Titration with 2:6-dichlorophenolindophenol is used to estimate vitamin C in a wide variety of foodstuffs, though the dye may react with substances besides ascorbic acid. Harris [1933] showed that dye-reducing substances can be produced by heating certain sugars with alkali. Johnson [1933] found with germinated peas that dye titrations indicated higher vitamin C contents than was shown by biological tests. In acid extracts part of the dye-reducing power was due to a substance which reduced the dye more slowly than ascorbic acid. Bacharach, Cook & Smith [1934] found an increase in the dye-reducing power of malt extract heated to 100° for ½–2 hr. McHenry & Graham [1935] obtained increases of 50–170% by heating certain vegetables for a few minutes at 58° or by standing these for a few hours at room temperature in 1% HCl. Ahmad [1935] found that boiling cabbage for 10 min. with 20% CCl_3COOH increased the apparent vitamin C content by nearly 200%. Reedman & McHenry [1938] found that the apparent vitamin C in heated potatoes resembled true vitamin C in both physiological and many chemical properties. Their suggestion that ascorbic acid might exist in combination with protein from which it was set free by heating was discredited by Harris & Olliver [1942], who suggested three possible causes for the results: (1) incomplete extraction from unheated tissues, (2) variations in sampling, (3) inhibition by heat of the ascorbic acid oxidase in the foods examined. The possible occurrence of non-specific dye-reducing substances in vegetable tissues was considered of little or no practical importance, although it was admitted that germinated seedlings, malt extract, yeast and fermented juices might repay further study.

During the last three years we have been accumulating evidence to show that the apparent vitamin C content of foods can become considerably higher than the true vitamin C value under manufacturing and storage conditions which may occur in practice. Preliminary reference to these findings was made by Wokes, Johnson, Duncan, Organ & Jacoby [1942] and by Wokes, Organ, Duncan & Jacoby [1943*a*]. The provisional term 'apparent vitamin C' was proposed to describe substances occurring in natural and processed foods which, although not possessing antiscorbutic activity, simulate vitamin C very closely in their chemical and physical properties, so that they are not distinguished from it by the indophenol titration as usually applied, and may therefore cause results to be too high. Methods of estimating true vitamin C in the presence of this apparent vitamin C have

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been described by Mapson [1943*a*, *b*] and by Wokes, Organ & Jacoby [1943*b*]. In this paper a brief account is given of the production and occurrence of apparent vitamin C in foodstuffs, and of some of its properties.

EXPERIMENTAL

Indophenol titrations

In our earlier experiments we used only the visual dye titration method essentially as described by Harris & Olliver [1942]. Later we also used the potentiometric method of Harris, Mapson & Wang [1942] with modifications. This proved invaluable both for deeply coloured products and for following dye reactions more precisely than is possible visually, and we are greatly indebted to these workers for details of the method prior to publication. Satisfactory agreement was obtained between visual and potentiometric results, provided that timing and rate of dye additions were carefully controlled as described by Wokes *et al.* [1943*b*].

Estimation of apparent vitamin C

This was carried out by a modification [Wokes *et al.* 1943*b*] of the method of Lugg [1942]. Lugg divided the various dye-reducing substances into three groups according to their rate of reaction with formaldehyde: (*a*) react readily at pH 1·5 and 3·5, (*b*) react readily at pH 3·5, slowly at pH 1·5, (*c*) do not react readily at either pH. Ascorbic acid was the only substance he had found in group (*b*); reductone occurred in group (*c*). Hence a basis was provided for differentiating ascorbic acid from reductone.

It has been shown by Mapson [1943*b*] and ourselves [Wokes *et al.* 1943*b*] that reductone and other components of apparent vitamin C react too rapidly with formaldehyde at pH 3·5 for satisfactory differentiation. We have obtained satisfactory results by treating mixtures of true and apparent vitamin C with formaldehyde at pH 4·5 for 6 min. This destroys the whole of the true vitamin C but little, if any, of the apparent vitamin C. Recovery experiments are made to correct for any apparent vitamin C destroyed in certain materials. The action of the formaldehyde is checked at the end of 6 min. by adding excess HPO_3 to bring the pH to about 1·0. To calculate true vitamin C the apparent vitamin C thus determined is subtracted from the total vitamin C estimated with similar timing and rate of dye additions.

Production of apparent vitamin C by heating with mineral acid

Potatoes, cabbages, carrots, germinated grains (wheat, barley and oats), grass and lucerne were treated with various concentrations of HCl, H_2SO_4, HPO_3 or CCl_3COOH in a water-bath at 90–95°, or occasionally on a sand-bath, water being added at intervals to make good any loss. With some materials (e.g. grass), heating on a sand-bath proved more effective. With HCl or H_2SO_4, 2% (v/v) of the concentrated acid gave the best results, producing as much apparent vitamin C as higher (up to 8%) concentrations of acid, but when less than 2% of concentrated acid was used the rate of production of apparent vitamin C fell rapidly with decreasing acid concentration. 1% HPO_3 was more effective than lower concentrations, but the rate of production of apparent vitamin C was still much slower than with 2% conc. HCl. The apparent vitamin C produced with 1% HPO_3 or with 0·25% conc. HCl was unstable to prolonged heating. When heating cabbage extract in 25% CCl_3COOH we were not able to obtain any increase in the dye titration values such as Ahmad [1935] described, but, on the contrary, as heating continued the titration value became less. Our cabbage extracts were prepared by grinding with quartz sand, with precautions to avoid destruction of ascorbic acid by oxidizing enzymes [vide Wokes & Organ, 1943]. Hence our findings indicate that the increase in the dye titration value observed by Ahmad when heating cabbage with 25% CCl_3COOH was due to extraction difficulties and not to production of apparent vitamin C. We were able to find only slight traces of apparent vitamin C in our cabbage extracts when examining these by the method of Wokes *et al.* [1943*b*]. On the other hand, when we heated germinated grains, grass and lucerne with acid, the dye titration figures steadily increased during several hours' heating (in contrast with the increase in 10 min. observed by Ahmad). The presence of apparent vitamin C was confirmed by the above method, although the amount found was sometimes less than the increase observed, and the possibility of formation of hydroxytetronic acid was not precluded. Nevertheless, ample evidence was obtained that the increase in the dye titration values was not due to any of the three possible causes mentioned by Harris & Olliver, viz. incomplete extraction, variation in sampling, or inhibition of ascorbic acid oxidase. A few representative results are given in Fig. 1.

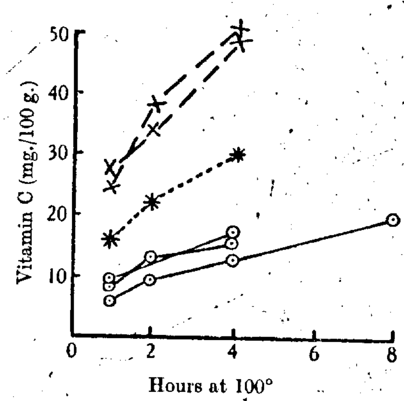


Fig. 1. Production of apparent vitamin C by heating with 2% conc. HCl or of conc. H_2SO_4 at 100°. Germinated grains, ⊙——⊙. Grass, ×——×. Lucerne, ✱·····✱.

Production of apparent vitamin C by heating without acid

In certain foods and food materials apparent vitamin C can be produced by heating to about 100° without addition of acid. This effect is strongly marked with malt extract, in which it is possible to increase the apparent vitamin C content as much as 10 times, but may also be observed with fruit syrups and other products. If true vitamin C be present, its destruction by heat may be masked by the simultaneous production of apparent vitamin C. This production of apparent vitamin C can take place at pH 4·5 as compared with a pH below 1 in the experiments with acid. A few typical results are given in Table 1.

Table 1. *Apparent and true vitamin C contents of foods, etc.*

Material	pH of material	Vitamin C content (mg./100 g.) Total	Apparent (a)	True	True as percentage of total
Fresh fruit and fruit preparations:					
Black currants, fresh	3·2	202	4	198	99
Black currant juice	3·2	140	5	135	96
Black currant syrup, freshly made	—	103	2	101	98
Black currant syrup after 8 weeks at room temp. in dark	—	96	4	92	96
Black currant syrup after 13 weeks at 27° in dark	—	58	3	55	94
Black currant syrup after 10 weeks at 37° in dark	—	68	8	60	88
Rose hip syrup after 12 months at 0–5°	3·7	156	20	136	88
Rose hip syrup after 12 months	3·6	101	69	32	31
Cherry juice, conc., after 5 years at 0–5°	3·8	47	45	2	4
Lemon juice, conc., after 18 months at 0–5°	1·7	193	42	151	78
Lemon juice, conc., after 4 years at 0–5°	1·7	27	21	6	22
Walnuts, unripe (*Juglans regia*), 2–3 g.	—	2619	1310	1309	50
Walnuts, unripe, 6–7 g.	5·2	2961	987	1974	67
Walnuts, unripe, 15–20 g.	3·9	2028	408	1620	80
Walnuts, unripe, after precipitation of tannin (*b*)	3·9	1909	400	1509	79
Dried fruits and vegetables:					
Dried bananas (*c*)	5·0	6	1	5	83
Dried carrots (*c*)	4·8	90	49	41	45
Dried potatoes (*d*)	4·5	27	4	23	85
Dried rose hip extract (mean of five samples) (*e*)	4·1	1300	50	1250	96
Dried spinach (*c*)	7·0	2	0·5	1·5	75
Dried tomatoes (*c*)	4·7	89	55	34	38
Malt extracts:					
Liquid malt extract, low diastatic power (D.P.)	4·7	3	(*f*)	0	0
Liquid malt extract, normal D.P.	4·7	12	(*f*)	0	0
Liquid malt extract, normal D.P.	4·8	5	(*f*)	0	0
Liquid malt extract after ½ hr. at 100°	4·8	12	(*f*)	0	0
Liquid malt extract after 2 hr. at 100°	4·8	20	(*f*)	0	0
Liquid malt extract after 9 months at 37°	3·6	103	(*f*)	0	0
Dried malt extract, low D.P. (*g*)	4·7	3	(*f*)	0	0
Dried malt extract, normal D.P. (*g*)	4·7	12	(*f*)	0	0
Sugars and molasses:					
Sugar, white (mean of three samples)	—	0	0	0	0
Sugar, brown (mean of four samples)	—	1	1	0	0
Golden syrup	—	27	27	0	0
Molasses, beet	—	18	18	0	0
Molasses, cane	—	11	11	0	0
Molasses, before charcoal treatment (*h*)	5·6	46	46	0	0
Molasses, after charcoal treatment	6·2	19	19	0	0
Molasses, beet concentrated preparation	5·4	222	(*d*)	0	0
Molasses, beet concentrated preparation	—	304	(*d*)	0	0
Molasses, cane concentrated preparation	—	196	(*d*)	0	0
Cocoa, chocolate:					
Cocoa, defatted, mixed sample	7·0	65	65	0	0
Cocoa, defatted, after precipitation of tannin	7·0	12	(*d*)	0	0
Chocolate, vitaminized	6·1	44	8	36	82
Chocolate, vitaminized	5·7	36	4	32	89
Miscellaneous:					
Beer, light draught	4·6	0·3	0·3	0	0
Beer, dark draught	4·4	1	1	0	0
Parsley (*i*)	7·0	220	40	180	82

ᴨparent vitamin C determined by method of Wokes *et al.* [1943*b*] checked occasionally by Mapson's method [1943*b*].
ethod of Mirimanoff & Mori [1940].
bout 4 years' storage in air-filled containers at room temperature.
weeks' storage in air-filled containers at room temperature.
ıge 4·0–4·2.
ıf the dye reductant in these preparations reacted with HCHO under the given conditions, but since it had produced by heating was considered not to be ascorbic acid.
ed by evaporation *in vacuo* of the corresponding liquid malt extract.
ıent normally used in refining sugar.
; extraction cyanide used to inhibit action of oxidizing enzymes and stabilize apparent and true vitamin C.

Development of apparent vitamin C during storage

Apparent vitamin C may be gradually formed during storage under normal conditions. The rate of formation is affected by temperature, moisture content and exposure to air and enzymic action.

(*a*) *Effect of storage temperature.* Fig. 2 summarizes some results obtained on malt extract, which show that the rate of formation of apparent vitamin C is affected by the storage temperature. At 37° increases of 12–103 mg./100 g. were observed during 268 days' storage. In the refrigerator at about 5° an increase of 46% was observed in 150 days.

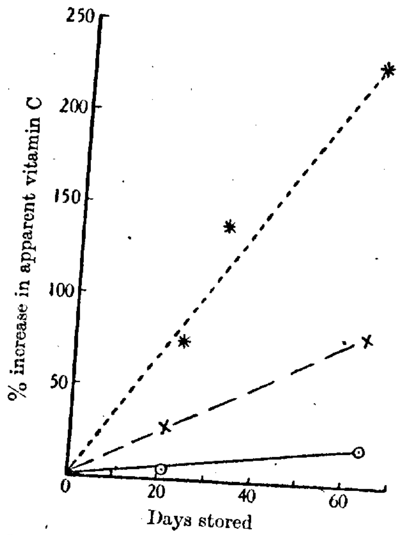


Fig. 2. Effect of storage temperature on rate of production of apparent vitamin C in malt extract. Increase at 37°, ✳·····✳. Increase at room temperature, ×——×. Increase at 0–5°, ⊙——⊙.

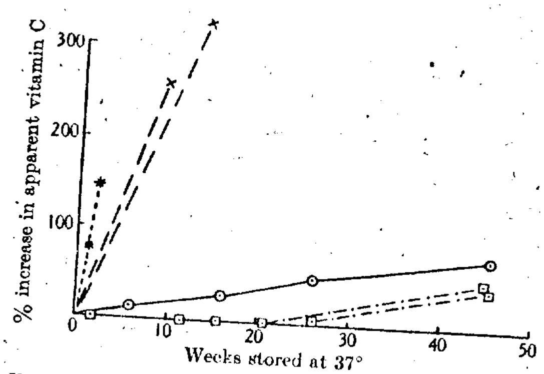


Fig. 3. Rate of production of apparent vitamin C in malt extract stored in air-filled containers at 37°, showing effect of enzyme and moisture content. Results on dried extract of low diastatic power (12 Lintner), ⊡-.-.-⊡. Dried extract of normal diastatic power (112 Lintner), ⊙——⊙. Liquid extract from which low D.P. dry extract prepared, ×——×. Liquid extract from which normal d.p. dried ext. prepared, ✳·····✳.

(*b*) *Effect of moisture content.* Removal of all but 1–2% of the moisture greatly retards the rate of production of apparent vitamin C. This is clearly shown by results of experiments on malt extract before and after drying. Rather less conclusive evidence was obtained, indicating that destruction of the malt enzymes also diminished the rate of production of apparent vitamin C (see Fig. 3).

In dried extracts of vegetables and fruits stored for 4 years at room temperature in air-filled containers there was marked development of apparent vitamin C, masking the gradual destruction of true vitamin C (see results with tomatoes in Fig. 4). Storage in nitrogen-filled containers reduced the rate of formation of apparent vitamin C but did not entirely prevent it.

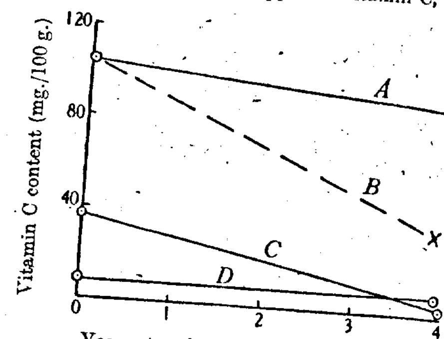


Fig. 4. Total and apparent vitamin C in dried fruit and vegetables stored in air at room temperature. *A*, total vitamin C in dried tomatoes. *B*, true vitamin C in dried tomatoes. *C*, total and true vitamin C in dried spinach. *D*, total vitamin C in dried bananas.

Occurrence of apparent vitamin C in miscellaneous materials

(*a*) *Cocoa and chocolate.* Apparent vitamin C has been found in considerable amounts in cocoa, and may therefore occur in chocolate used as a carrier of the vitamin (Table 1). When the true vitamin C gradually disappears during storage, the apparent vitamin C remains, and if no correction is made for its presence the error increases in magnitude. The apparent vitamin C is not entirely tannin, since it is not completely precipitated by the method of Mirimanoff & Mori [1940].

(*b*) *Fruits and fruit syrups.* Fresh fruits appear on the whole to contain only small amounts of apparent vitamin C, which does not usually exceed 10% of the total vitamin C found. Unripe walnuts are an exception; in some species the apparent vitamin C may be 80% of the total [Melville, Wokes & Organ, 1943].

When fruit juices are heated or subjected to prolonged storage more apparent vitamin C may be produced, so that it may eventually form the greater part of the total vitamin C found by the usual dye titration (Table 1). Some of this may be reductic acid formed by the action of the fruit acids on pectin or other constituents.

(*c*) *Sugar, syrup and molasses.* Demerara and other coloured sugars contain traces of apparent vitamin C, which may produce an appreciable effect on the total vitamin C content of fruit syrups. Some samples of table syrup contain more apparent vitamin C. This is also found in molasses, from which very concentrated preparations can be obtained. In the process of sugar refining the apparent vitamin C is presumably produced by the treatment with mineral acid, and partially removed by subsequent treatment with charcoal.

(*d*) *Vegetables.* Significant amounts of apparent vitamin C have not been found in common raw vegetables, but may be produced by cooking at a high temperature (e.g. roasting or frying), and also occur in some herbs such as sorrel and parsley.

(*e*) *Beer.* Traces of apparent vitamin C, comparable with the total vitamin C contents of South African beers reported by Levy & Fox [1935], have been found in English draught beer, especially in the dark variety.

Properties of apparent vitamin C

(*a*) *Temperature coefficient.* The rate of production of apparent vitamin C increases with rise in temperature. Between 0 and 37° the temperature coefficient for 10° rise is of the order of 2. At 100° a sharp rise in the rate occurs.

(*b*) *Effect of* pH *on rate of formation and stability.* Apparent vitamin C develops as rapidly at pH 4–5 as at pH 1, and in part at least is unstable in 5% HPO_3 solution (pH below 1) as well as in less acid aqueous solutions. Its decomposition may be retarded by bubbling nitrogen through the solution to expel air, and by storage in the dark in nitrogen-filled containers; also by the use of cyanide.

(*c*) *Effect of oxidizing enzymes.* These, including ascorbic acid oxidase, were extracted from outer cabbage leaves by the method of Szent-Györgyi [1931], from cauliflower inner florets by the method of Hopkins & Morgan [1936] and from unripe tomato skins by the method of Wokes & Organ [1943], and tested on apparent vitamin C obtained either at a pH of about 1 (e.g. beet and cane molasses, acid-heated extracts of various foods) or at a pH of about 5 (e.g. dehydrated foods, malt extract). Freshly prepared solutions in buffers of pH 2–6 were mixed with the enzyme extracts at room temperature and apparent vitamin C determined at intervals. The enzymes destroyed apparent vitamin C under the same conditions as they destroyed real vitamin C.

(*d*) *Effect of oxidizing agents.* Practically the whole of the apparent vitamin C in different foods can be destroyed within 2–3 min. by treatment with 0·1% hydrogen peroxide or sodium percarbonate at 37°. This treatment also destroys true vitamin C. With lower concentrations less of the true or apparent vitamin C was destroyed. Even 1:10^8 dilution of H_2O_2 produced an appreciable effect. The rate of destruction was most rapid in the first minute. It did not appear possible to differentiate between true and apparent vitamin C by such means.

(*e*) *Effect of ultra-violet light.* Griffiths [1943] suggested that reductones might be formed by the action of ultra-violet light on substances in the atmosphere. Solutions of dehydrated foods, in which it was known that apparent vitamin C could be developed during prolonged storage, were irradiated (mercury vapour lamp) at pH 4–6 in covered Petri dishes for several hours, screens being used to cut out short wave-length rays not present in sunlight, without development of apparent vitamin C. Solutions of foods in which the apparent vitamin C was already present were irradiated under similar conditions, and the apparent vitamin C was gradually destroyed, having almost entirely disappeared after 3–4 hr. irradiation [Wokes, 1943]. Whilst ultra-violet light did not produce any apparent vitamin C in these dehydrated foods, the position may be quite different in natural foods, such as fruits, which are exposed to sunshine and contain various pigments and enzyme systems.

(*f*) *Spectroscopic evidence.* Dr R. A. Morton kindly examined spectroscopically three of our most concentrated sources of apparent vitamin C obtained from malt extract by prolonged incubation, and from beet and cane molasses respectively. The treatment which these had undergone precluded the presence of true vitamin C, and the absence of antiscorbutic properties was also shown by physiological tests. The incubated malt extract showed a very distinct maximum at about 280 mμ (Fig. 5). Maxima in the beet

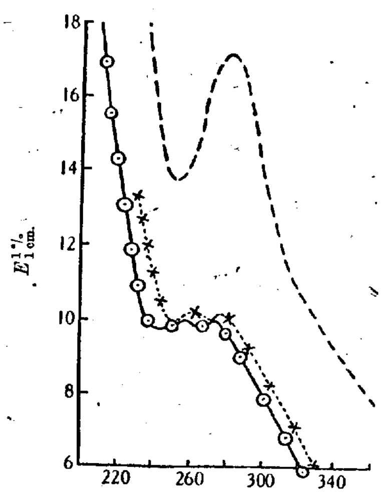


Fig. 5. Absorption spectrograms of concentrated preparations of apparent vitamin C obtained from: malt extract, – – – –; beet molasses, × ----- ×; cane molasses, ⊙——⊙.

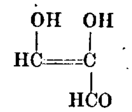
molasses at about 276 and 264 mμ, and in the cane molasses at about 275 and 256 mμ, were obscured by general absorption, due to the dark colour. When this dark colour was removed by treatment with charcoal, the apparent vitamin C was not recovered. Attempts to obtain colourless extracts suitable for spectroscopic examination by shaking or Soxhlet extraction with methanol or ethanol or by prolonged shaking with ether proved unsuccessful.

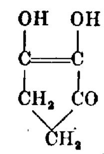
DISCUSSION

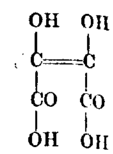
The production of non-specific dye reductants by heating different foodstuffs with or without acid has been mentioned by a number of previous workers. Very little was known of the nature and properties of these reductants and they were generally assumed not to affect the vitamin C value of foods as determined by dye titrations, either because they were not present in significant amounts or because they reacted more slowly with the dye so that their interference was obviated by carrying out the titration very rapidly, the end-point being reached in a minute or less. However, some doubt has been cast on this assumption by the recent findings of Hochberg, Melnick & Oser [1943] and Taylor [1943] that, when the end-point is reached still more rapidly by extrapolation of photoelectric data, the results may be appreciably lower than those given by the ordinary visual methods. Taylor [1943] found the total vitamin C in unripe walnuts to be about 13% less by the more rapid method, and similar discrepancies occurred with other foods.

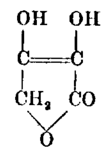
Wokes *et al.* [1943*b*], using a potentiometric method, have shown that the vitamin C value obtained for samples of dehydrated carrots, in which much apparent vitamin C had developed during 4 years' storage, was increased as longer intervals were allowed between the dye additions, thus allowing more time for the apparent vitamin C to react. With additions every 15 sec. the result was about 40 mg./100 g.; with additions every 8 min. the result was over 100 mg./100 g. It was suggested that this was due in part to enol-keto tautomerism. Apparent vitamin C was thought to exist in the solutions undergoing titration as a mixture of two isomers. Only one of these reacts with the dye. As the dye is added it removes the active isomer, and more of this is formed from the inactive isomer to restore the equilibrium. Thus the time effect is due to the rate of enol-keto tautomerism as well as to the rate of reaction of the active form with the dye.

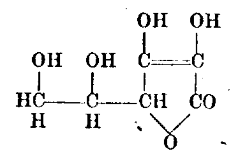
In the present investigation it has been found that apparent vitamin C can be formed under much less drastic conditions, and occurs more widely and in greater proportions than was previously supposed. Moreover, its components resemble true vitamin C closely (1) in their susceptibility to oxidation by very low concentrations of inorganic or organic peroxides—an oxidation which is accelerated by enzyme extracts containing ascorbic acid oxidase, (2) in their removal by activated charcoal and general behaviour towards adsorbents, and (3) in their reduction of iodine, ammoniacal silver nitrate or alkaline copper solutions (Benedict's, Fehling's) in the cold. Some of the reductants in apparent vitamin C are gradually destroyed on standing in HPO_3 solution at room temperature, but may be stabilized by nitrogen or cyanide; others are stable in HPO_3 solution. They also differ from vitamin C in possessing no antiscorbutic activity and in being more stable towards heat and towards formaldehyde at pH 4–5 [Wokes *et al.* 1943*b*]. They should resemble ascorbic acid closely in chemical constitution and contain the active dienol and carbonyl groups. These are present in reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid, four compounds closely related to ascorbic acid, as shown by comparison of their formulae.


Reductone

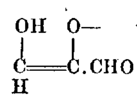

Reductic acid


Dihydroxymaleic acid


Hydroxytetronic acid


Ascorbic acid

Reductone is formed, amongst other products, when carbohydrates are treated with alkali. Such treatment may produce a variety of enolic compounds, hence the 'reductones' which Mapson prepared may have contained other dye reductants besides pure reductone, especially as their properties differed in certain respects (e.g. stability towards HPO_3) from those of our apparent vitamin C. Martius & Euler [1934] showed that reductone and dihydroxymaleic acid react much more slowly with the dye when the pH is >5, and suggested that reductone could dissociate into two tautomeric ionized forms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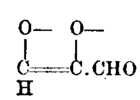

Snow & Zilva [1938], when describing their experiments with ascorbic acid oxidase on reductone, suggested that their findings might be explained by the high dye-reducing power of one of these isomers, the concentration of which would be controlled by the velocity of the tautomeric conversion. A similar explanation is suggested for the variations we have observed in the reaction velocity of the dye with apparent vitamin C in different foodstuffs. Reductone has an absorption maximum at 287 mμ above pH 5·7 and at 268 mμ at pH 4·0 or lower, indicating a change in the proportion of the two isomers in this pH range which may be related to the difference in properties of apparent vitamin C in foods of pH above or below this range. The maximum at 268 mμ would be in good agreement with the maximum of 264 mμ found in our beet molasses at a similar pH, but the evidence for the presence of reductone is not conclusive. Euler & Martius [1933] stated that reductone has no antiscorbutic activity.

Reductic acid was obtained by Reichstein & Oppenauer [1933] by heating beet sugar pectin or various carbohydrate derivatives with mineral acid, treatment like that which produced apparent vitamin C in our molasses. Reductic acid has an absorption maximum at 280mμ at pH 6 and at 264mμ at pH 3 [Mayer, 1937]. The spectroscopic data on our materials do not preclude the presence of reductic acid, but the fact that the apparent vitamin C was not completely destroyed on standing in HPO_3 [Wokes *et al.* 1943*b*] suggested that the latter contained at least two different substances.

Dihydroxymaleic acid is formed when tartaric acid is oxidized by H_2O_2 in presence of small quantities of ferrous salts in sunlight. Szent-Györgyi [1939] states that it is quite common in plants, where it probably serves as a hydrogen transmitter analogous to ascorbic acid, being reversibly oxidized by an oxidase similar to ascorbic acid oxidase. Dihydroxymaleic acid, like ascorbic acid, is rapidly oxidized by traces of peroxides, but unlike ascorbic acid is unstable in acid solutions, in which it resembles part of the apparent vitamin C in our materials. Solutions of dihydroxymaleic acid in HPO_3 are not stabilized by nitrogen or cyanide. Such stabilization might be effected in the presence of certain food extracts, but our experiments in this direction have so far proved unsuccessful. Potentiometric studies of its rate of reaction with the dye confirm Szent-Györgyi's suggestion that in aqueous solution it is subject to enol-keto tautomerism. The maximum light-absorption for dihydroxymaleic acid in water is at 290mμ. This differs appreciably from the maximum at 280mμ in our incubated malt extract, and the maximum at 275-276mμ in the molasses, but the possibility must be considered that the acid in the molasses may have shifted the dihydroxymaleic acid maximum from 290 mμ to a lower wave-length. The presence of dihydroxymaleic acid in our materials is therefore not excluded. The destruction of the non-specific reductant in these materials by our enzyme extracts may have been due to their containing dihydroxymaleic acid oxidase, which Banga & Philipot [1938] found in many plants. Dihydroxymaleic acid is not antiscorbutic [Dalmer, 1934].

Hydroxytetronic acid is chemically of interest as being the parent substance from which ascorbic acid is probably formed. It resembles ascorbic acid closely in its rate of reaction with HCHO and with the indophenol dye. Its maximum absorption at 246mμ is appreciably lower than the nearest maximum of 256mμ in any of our materials, and would presumably tend to become still lower in presence of acid. Its occurrence in our materials seems less likely than that of reductic or dihydroxymaleic acids. Hydroxytetronic acid is not antiscorbutic [Micheel & Jung, 1934].

Other possible constituents of apparent vitamin C. It appears from spectroscopic evidence, and from the effects of HPO_3 and HCHO at different pH [Wokes *et al.* 1943*b*], that apparent vitamin C frequently contains more than one non-specific reductant. In addition to the reductants mentioned above, enolic substances may be present which reduce the dye fairly rapidly but which are less closely related to ascorbic acid. Certain German workers [cf. Enders, 1938; Luers, 1937] have described the occurrence and gradual development, in malted barley extracts and beer during storage, of dye reductants which they state are formed by condensation of sugars and amino-acids with evolution of CO_2. These reductants are stated not to reduce Fehling's solution, which indicates that their chemical nature differs more widely from that of ascorbic acid than does that of the above four reductants.

SUMMARY

1. The provisional term 'apparent vitamin C' is proposed to describe a group of substances occurring in foods and closely resembling ascorbic acid in chemical and physical properties (as well as in behaviour towards oxidizing enzymes), so that they are not distinguished from vitamin C by the dye titration generally employed.

2. These substances probably all contain the dienol group, and may include reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid. They undergo enol-keto tautomerism, which causes their rate of reaction with the dye, and other properties, to be affected by the pH, the range 4-6 appearing to be critical.

3. They differ from ascorbic acid in being produced by the action of heat on certain constituents (e.g. pectins, carbohydrates) in foods and also (except hydroxytetronic acid) in their rate of reaction with formaldehyde under given conditions [Wokes *et al.* 1943*b*]. These differences in behaviour have formed the basis of methods of estimating apparent vitamin C in foodstuffs.

4. Apparent vitamin C has been found in germinated grains, malt extract, cocoa, chocolate, dehydrated fruits and vegetables, beer, and in certain fruits and herbs such as walnuts, parsley and sorrel. Particularly high contents have been found in unripe walnuts, in concentrated preparations from beet and cane molasses, and in fruit juices and dried foods stored for several years under normal conditions.

The apparent vitamin C content of processed and dehydrated foods may gradually increase during storage under normal conditions, and thus obscure loss of true vitamin C in storage experiments.

We are indebted to Dr L. J. Harris and his colleagues for advice and criticism, to Dr R. A. Morton for spectroscopic examination of various samples, to Mr H. C. Powers, chief chemist, Messrs Tate and Lyle's Thames Refinery, for samples of molasses, to Messrs W. J. Bush and Co. for samples of fruit juices and syrups, and to Dr F. Bergel for specimens of reductic and hydroxytetronic acid.

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REFERENCES

Ahmad, B. [1935]. *Biochem. J.* **29**, 275.
Bacharach, A. L., Cook, P. M. & Smith, E. L. [1934]. *Biochem. J.* **28**, 1038.
Banga, I. & Philipot, E. [1938]. *Hoppe-Seyl. Z.* **258**, 147.
Dalmer, O. [1934]. *Dtsch. med. Wschr.* **60**, 1200.
Enders, C. [1938]. *Kolloidzschr.* **85**, 74.
Euler, H. v. & Martius, C. [1933]. *Liebigs Ann.* **505**, 73.
Griffiths, J. G. A. [1943]. *Nature, Lond.*, **152**, 163.
Harris, L. J. [1933]. *Nature, Lond.*, **132**, 27.
—— Mapson, L. W. & Wang, Y. L. [1942]. *Biochem. J.* **36**, 183.
—— & Olliver, M. [1942]. *Biochem. J.* **36**, 155.
Hochberg, M., Melnick, D. & Oser, B. L. [1943]. *Industr. Engng Chem.* (Anal. ed.), **15**, 182.
Hopkins, F. G. & Morgan, E. J. [1936]. *Biochem. J.* **30**, 1446.
Johnson, S. W. [1933]. *Biochem. J.* **27**, 1942.
Levy, L. F. & Fox, F. W. [1935]. *S. Afr. med. J.* **9**, 181.
Luers, H. [1937]. *Münch. brautech. Zbl.* **36**, 11.
Lugg, J. W. H. [1942]. *Aust. J. exp. Biol. med. Sci.* **20**, 273.
Mapson, L. W. [1943*a*]. *Nature, Lond.*, **152**, 13.
—— [1943*b*]. *J. Soc. chem. Ind., Lond.* **62**, 223.
Martius, C. & Euler, H. v. [1934]. *Biochem. Z.* **271**, 9.
Mayer, N. [1937]. *J. Chim. phys.* **34**, 109.
McHenry, E. W. & Graham, M. [1935]. *Biochem. J.* **29**, 2013.
Melville, R., Wokes, F. & Organ, J. G. [1943]. *Nature, Lond.*, **152**, 447.
Micheel, F. & Jung, F. [1934]. *Ber. dtsch. chem. Ges.* **67 B**, 1660.
Mirimanoff, A. & Mori, M. [1940]. *Schweiz. Apoth Ztg.* **78**, 685.
Reedman, E. J. & McHenry, E. W. [1938]. *Biochem. J.* **32**, 85.
Reichstein, T. & Oppenauer, R. [1933]. *Helv. chim. Acta*, **16**, 988.
Snow, G. A. & Zilva, S. S. [1938]. *Biochem. J.* **32**, 1926.
Szent-Györgyi, A. V. [1931]. *J. biol. Chem.* **90**, 384.
—— [1939]. *Oxidation, Fermentation, Vitamins, Health and Disease.* Baltimore: Williams and Wilkins.
Taylor, A. McM. [1943]. *Biochem. J.* **37**, 54.
Wokes, F. [1943]. *Nature, Lond.*, **152**, 328.
—— Johnson, E. H., Duncan, J., Organ, J. G. & Jacoby, F. C. [1942]. *Quart. J. Pharm.* **15**, 314.
—— & Organ, J. G. [1943]. *Biochem. J.* **37**, 259.
—— —— Duncan, J. & Jacoby, F. C. [1943*a*]. *Nature, Lond.*, **152**, 14.
—— —— & Jacoby, F. C. [1943*b*]. *J. Soc. chem. Ind., Lond.* **62**, 232.

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Proc. Soc Exp Biol and Med 47(2): 490-492. 1941

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Effects of Clinical Doses of Phenobarbital on Blood and Urine Ascorbic Acid in Human Subjects.

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That barbituric acid derivatives were effective in stimulating ascorbic acid excretion in rats was demonstrated by Longenecker and his co-workers.[1] Within 5 days the administration of sodium phenobarbital caused an increase of 15 times in the urinary excretion of ascorbic acid.

This experiment records the effects of clinical doses of phenobarbital on the plasma, whole blood, and urinary values of ascorbic acid in human subjects. Although some other drugs are equally effective in stimulating ascorbic acid excretion in animals, phenobarbital was chosen because it is so commonly used in clinical medicine. It should be recalled that a daily dose of 20 mg of sodium phenobarbital was given by King and his co-workers to a 250 g rat, whereas we administered 180 mg to a 70K subject.

In rats, it was shown that 5 days were required to stimulate the maximum excretion of ascorbic acid, so a 5-day period of phenobarbital administration was chosen for the human subjects. The 180 mg daily dose did affect the subjects in so much that dizziness and diplopia were notable on the 5th day.

Plan of Experiment. Three subjects were used. Subject A was a well developed male weighing 170 pounds, subject B a well developed male weighing 185 pounds, and subject C a normal adult female weighing 125 pounds. The following diet was consumed each day during the entire experiment:

Food	g	Food	g	Food	g
Am. cheese	20	Figs, dried	30	Pork	75
Bacon	10	Flour	3	Puffed wheat	15
Beef	75	Grape jelly	30	Ripe olives	30
Butter	45	Grape juice	100	Soda crackers	12
Cream	170	Jello	16	Sugar	13
Dates	20	Limas, dried	20	Walnuts	15
Eggs	2	Macaroni	10	W. W. bread	120
Evap. milk	200	Peas, dried	20	Total calories	3021

Total calories in individual diets were adjusted by varying the amount of bread, milk, cream, butter, and flour according to the

[1] Longenecker, H. E., Fricke, H. H., and King, C. G., *J. Biol. Chem.*, 1940, **135**, 497.

individual preferences of the 3 subjects. During the month of the experiment the weights of the subjects did not change.

The vitamin C free diet was supplemented with 25 mg of pure ascorbic acid administered with the morning meal. The constancy of the diet ruled out variations in ascorbic acid excretion due to changes in pH of urine. Ascorbic acid and phenobarbital were taken at approximately the same time each day. It was hoped to arrange the ascorbic acid intake so that blood, and urinary changes would be evident. The subjects voided frequently enough so that urine remained in the bladders of the subjects for only a short period of time.[2] The urine volumes were quite constant, that of subjects A and B averaged 1250 cc daily, while that of subject C averaged 889 cc daily. The subjects performed the same work daily either in the hospital or laboratory. Following a 10-day control period, phenobarbital was taken for a period of 5 days. A second control period followed the drug period.

Chemical Methods. Fasting daily specimens of blood were taken. The plasma and whole blood methods of Mindlin and Butler[3] and Butler and Cushman[4] were used to determine the ascorbic acid values.

The urine ascorbic acid was determined on 24 hour specimens by the method of Roe and Hall[5] adapted to the Evelyn photoelectric colorimeter. Recovery experiments on this method gave 90% to 99% recovery of added ascorbic acid.

Results are recorded in Table I.

It is evident that there was a very slight but definitely steady decline in whole blood, plasma, and urinary excretion of ascorbic acid during the 3 weeks of observation in all 3 subjects. In both male subjects, no significant change took place in the ascorbic acid metabolism during the administration of phenobarbital. In the one female subject, there appeared a slight but significant rise in ascorbic acid excretion and decrease in the whole blood values during administration of phenobarbital. We cannot explain this observation.

If ascorbic acid were used by the human being in detoxifying phenobarbital as Longenecker, *et al.,* believe occurs in the rat, we would expect a decrease in the blood value for ascorbic acid and a decrease in urinary output during phenobarbital administration to man. It is possible that if on the basis of weight the dosage to

[2] Sherry, S., and Friedman, G. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 707.

[3] Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

[4] Butler, A. M., and Cushman, M., *J. Clin. Invest.*, 1940, **19**, 459.

[5] Roe, J. H., and Hall, J. M., *J. Biol. Chem.*, 1939, **128**, 329.

TABLE I.
Ascorbic Acid Concentration of Whole Blood, of Plasma and of Urine.

Date 1941	A: Whole blood mg%	A: Plasma mg%	A: Urine mg%	A: Urine 24 hr mg	B: Whole blood mg%	B: Plasma mg%	B: Urine mg%	B: Urine 24 hr mg	C: Whole blood mg%	C: Plasma mg%	C: Urine mg%	C: Urine 24 hr mg
2-5	.84	.40	.61	5.20	.63	.22	.38	4.40				
2-6	1.22	.46	.45	5.48	1.12	.31	.32	4.13				
2-7	1.27	.43	.46	4.80	.86	.33	.28	3.47				
2-8	.99	.43	.39	4.84	1.20	.40	.34	2.43				
2-9	1.12	.58	.35	3.94	.89	.55	.20	1.86	1.12	.58	.34	2.90
2-10	.96	.40	.69	7.54	1.07	.34	.58	6.84	1.20	.54	.89	6.30
2-11	1.37	.42	.48	6.73	.63	.39	.31	3.91	1.37	.60	.66	2.15
2-12	1.36	.40	.48	4.90	1.04	.37	.27	4.90	1.25	.55	.43	3.30
2-13	.79	.34	.46	2.59	.71	.36	.44	5.20	1.22	.50	.45	4.50
2-14	.76	.30	.43	4.18	.73	.30	.31	3.85	.92	.51	.40	3.89
2-15	.77	.32	.50	5.76	.64	.21	.35	3.89	1.00	.47	.43	4.65
Phenobarbital gr iii h.s.												
2-16	.56	.32	.19	2.31	.62	.23	.12	2.18	.68	.52	.28	3.43
2-17	.86	.26	.25	3.53	.76	.14	.27	2.50	.95	.39	.47	3.60
2-18	.80	.31	.49	6.32	.66	.19	.42	5.75	.80	.38	.68	5.60
2-19	.63	.16	.41	5.27	.57	.14	.28	5.34	.70	.32	.53	5.68
2-20	.73	.25	.31	4.30	.52	.16	.27	3.70	.56	.35	.56	4.92
Drug discontinued												
2-21	1.10	.24	.24	2.64	.72	.23	.22	2.92	1.03	.33	.38	3.40
2-22	.60	.20	.32	5.51	.65	.20	.25	3.40	.73	.38	.54	3.80
2-23	.64	.27	.25	3.47	.53	.23	.20	5.30	.74	.32	.36	3.97
2-24	.67	.25	.35	3.50	.67	.15	.34	3.59	.81	.32	.52	3.30
2-25	1.05	.20	.33	4.95	1.11	.15	.24	2.40				
2-26	.50	.16	.24	3.66	.46	.16	.24	2.40				
2-27	.64	.16	.13	1.15	.64	.13	.24	2.40				
Avg												
1st control	1.04	.41	.48	5.09	.87	.34	.34	4.08	1.15	.54	.52	3.96
Drug	.72	.26	.33	4.35	.63	.17	.27	3.89	.74	.39	.50	4.65
2nd control	.74	.21	.27	4.55	.68	.18	.25	3.20	.83	.34	.45	3.62

humans had been comparable to that given by Longenecker to rats some effect would have been noted. It is possible the drug was conjugated with ascorbic acid in the urine, but this does not occur in rats and one would expect in this case some reduction in ascorbic acid values in whole blood or plasma.

Summary. There was no significant change in whole blood, plasma, or urinary excretion of ascorbic acid following administration of 180 mg of phenobarbital daily to human subjects. Twenty-five mg of crystalline ascorbic acid daily is insufficient to maintain whole blood or plasma values when the subjects take an ascorbic acid-free diet.

Krebsforsch 76(1) 1-7

Z. Krebsforsch. 76, 1—7 (1971)

Antitumor Potency of Ascorbic, Dehydroascorbic or 2, 3-Diketogulonic Acid and Their Action on Deoxyribonucleic Acid

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Received January 19, 1971; accepted February 19, 1971

Summary. Three nutritively significant enediol compounds mentioned in above title can inhibit sarcoma-180 growth. The inhibition by ascorbic acid is enhanced by cupric ions. These enediols depolymerize DNA, particularly in co-operation with copper. Dehydroascorbic acid is able to break denatured DNA and to bring about single strand scission for native one. A mixture of dehydroascorbate and Cu decomposes apurinic acid and liberates deoxy-cytidylic acid from it. Only the oligo-form pf pyrimidine tetra- and penta-nucleotides is disintegrated by this reagent. The results were briefly discussed in relation to metabolic carcinostasis and to the formulation of Yamafuji effect causing cellular differentiation and anomalization.

Zusammenfassung. Die im obigen Titel erwähnten, drei nutritiv bedeutenden Endiol-Verbindungen können Sarkom-180 hemmen. Die hemmende Wirksamkeit von Ascorbinsäure wird durch Cupri-Ionen verstärkt. Diese Endiolen depolymerisieren DNS, besonders unter Mitwirkung von Kupfer. Dehydroascorbinsäure besitzt die Fähigkeit, denaturierte DNS zu brechen und eine einstrangige Spaltung für native Nucleinsäure zu bewirken. Eine Mischung von Dehydroascorbat und Cu zersetzt Apurinsäure und trennt daraus Deoxy-cytidylsäure. Nur die Oligo-Form von Pyrimidin-Tetra- und Penta-Nucleotiden wird durch dieses Reagens desintegriert. Die Ergebnisse wurden im Zusammenhang mit der metabolischen Carcinostasis und der Formulierung des cellulären, Differenzierung und Anomalisation verursachenden Yamafuji-Effekts kurz diskutiert.

Introduction

It has been previously demonstrated that catecholamines possessing enediol-group inhibit tumour and react with nucleic acids (Yamafuji *et al.*, 1970). As vitamin C belongs to the reductone, we assumed that this acid and some of its metabolic products can exhibit similar properties. Although we already reported a sarcoma-inhibition by dehydroascorbic acid (Nakamura and Yamafuji, 1968), we have now performed more detailed and extendend investigations in connection with the behaviour of ascorbic or diketogulonic acid.

Materials and Methods

Antitumouric Capacity of Ascorbic, Dehydroascorbic and 2, 3-Diketogulonic Aacids. Male ddN-mice were obtained from Breeding Center as described before (Yamafuji *et al.*, 1971). In the present study, ascites from the mouse in 7 to 9 days after injecting sarcoma-180 was used as implanting material. The concentration of tumour cells was regulated to 2×10^6/0.2 ml with Ringer solution and the implantation subcutaneously carried out as stated in the preceding paper (Yamafuji and Murakami, 1968). The enediol solution was injected every other day from 30 hours after implanting tumour and the injection of Ringer solution of the same pH conducted in the control. The period of test was 2 weeks, during which mice were fed with a diet from Oriental Yeast Co. at 25° C.

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Dehydroascorbic acid used in this work was prepared from ascorbic acid (100% purity) with iodine as mentioned previously (Nakamura and Yamafuji, 1968). The preparation consisted of 66% dehydroascorbic acid, 10% 2,3-diketogluconic acid, 2% L-ascorbic acid and 22% other decomposition products. To prepare 2,3-diketogulonic acid, ascorbic acid was oxidized with K-iodate according to the method of Kagawa and Takiguchi (1962). The analysis with 2,4-dinitrophenylhydrazine (Roe *et al.*, 1948) indicated that the product is composed of 64% diketogulonic and 10% dehydroascorbic acid. As preliminary trials showed that an injecting solution of pH 5.5 is favourable for the antitumour tests of these acidic compounds, the Ringer solution of the reductones was regulated to this acidity with 0.01 M acetate buffer. The drugs were applied in the neighbourhood of transplanted sarcoma.

Breakage of DNA by Ascorbic, Dehydroascorbic and 2,3-Diketogulonic Acids. In order to measure the viscosity-lowering, calf thymus DNA was dissolved in 0.15 M NaCl + 0.015 M Na-citrate (SSC) and mixed with respective reagents. The final concentration of nucleic acid, enediols, Cu-sulphate and phosphate buffer (pH 7.0) was 100 µg/ml, 5×10^{-3} M, 3×10^{-4} M and 0.1 M respectively. Temperature was 37° C. Effect of anions was examined in the dehydroascorbate-solution containing 10^{-3} M Cu-salts.

To prove the cleavage of long native DNA, a mixture of thymus nucleic acid (50 µg/ml), 5×10^{-3} M dekydroascorbic acid, 3×10^{-4} M $CuSO_4$ and 4×10^{-2} M phosphate buffer (pH 7) was maintained at 37° C for 60 min. After dialysing against SSC for 44 hours at 5° C, a sucrose gradient centrifugation was applied to the solution of pH 7 at 30,000 rpm for 4 hours by Spinco L-ultracentrifuge with SW 39 L roter. For examining whether single strand scission occurs, the ultracentrifugation of the solution incubated without Cu was performed at pH 12.8. To investigate the splitting of denatured nucleic acid, DNA was heated at 100° C for 10 min and cooled rapidly. After reacting with dehydroascorbate (or + Cu), the dialysed neutral solution was fractionated with Spinco-centrifuge.

Decomposition of Pyrimidine Nucleotide Cluster by Dehydroascorbic Acid. A reaction mixture containing apurinic acid (200 µg/ml) prepared with HCl (Tamm *et al.*, 1952), 10^{-2} M dehydroascorbate, 10^{-3} M $CuSO_4$ and 5×10^{-2} M phosphate buffer of pH 7 incubated at 37° C for 12 hours. The solution was gel-filtered through Sephadex G-50 column and the absorption spectra of filtrate measured at 260 mµ. At the same time, the 260 mµ-spectrum of the mixture dehydroascorbate + copper was estimated after the incubation, and the value obtained was subtracted from the test one.

With the aim of identifying the decomposition product, the incubated reaction mixture of the same composition as mentioned above was dialysed with a cellulose tubing (Size 20/32 of Visking Co.) against water for 48 hours at 5° C. The outside solution was freeze-dried and paper-chromatographed with a solvent, isopropanol 170: HCl 41. For the purpose of determining the degree of decomposition, pyrimidine nucleotide was prepared with H_2SO_4 by the method of Spencer and Chargaff (1961). The preparation from 40 mg thymus DNA was neutralized, mixed with 0.125 M dehydroascorbic acid and 0.0125 M $CuSO_4$. After keeping at 37° C for 12 hours, the solution was column-chromatographed with DEAE-cellulose. Apyrimidinic acid and purine nucleotide were also prepared according to the process proposed by Takemura (1959) and by Habermann *et al.* (1963) respectively. It was examined whether these compounds are decomposed by dehydroascorbate, applying the procedures similar to those employing for apurinic acid and pyrimidine nucleotide.

Results

Antitumouric Capacity of Ascorbic, Dehydroascorbic and 2,3-Diketogulonic Acids. The data of animal tests are shown in the table.

The inhibition ratios calculated indicate that ascorbic, dehydroascorbic and diketogulonic acids have a fairly strong sarcoma-180-hindering potentiality. The relatively low activity of ascorbic acid could be strengthened by copper salt. The body weight of mice was not influenced by the enediol-injection.

Breakage of DNA by Ascorbic, Dehydroascorbic and 2,3-Diketogulonic Acids.

Table. *Antitumour activity of enediols*

Agent	No. of mice	Place of injection	Dose in each injection (mg/kg)	Times of injection	Average Tumour weight (g)	Inhibition ratio (%)
Ascorbic acid (AA)	10	Subcutaneous	150 (AA)	5	2.1	46.5
Ascorbic acid + $CuSO_4$	10	Subcutaneous	150 (AA) + 5.4 (Cu)	5	1.2	69.2
Control	10	Subcutaneous	—	5	3.9	—
Dehydroascorbic acid	10	Subcutaneous	120	6	0.5	88.1
Control	10	Subcutaneous	—	6	4.2	—
2,3-Diketogulonic acid	10	Subcutaneous	115	5	2.0	54,5
Control	10	Subcutaneous	—	5	4.4	—

concentration of 5×10^{-3} M, but it can be remarkably enhanced by a small amount of copper.

The curves in Fig. 2 revealed further that the kind of anions in Cu-salts has no influence upon the viscosity of DNA. In view of the fact that copper exists as a normal constituent in organisms, there is the possibility that the DNA-depolymerization occurs powerfully after injecting enediols.

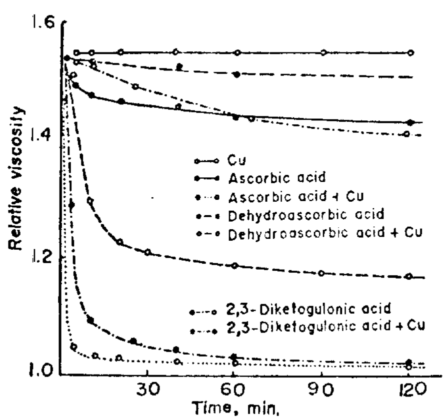

Fig. 1. Viscosity of DNA in solutions of enediols ± $CuSO_4$

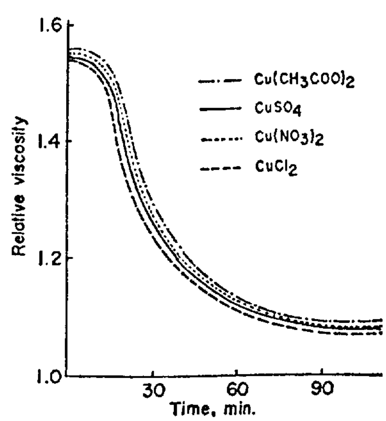

Fig. 2. Viscosity of DNA in dehydroascorbate solutions with various Cu-salts

As depicted in Fig. 3, a gradient centrifugation at pH 7 confirmed actually that a double strand-cleavage takes place in the portion of comparatively high mole-alone. Dehydroascorbate of 5×10^{-3} M slightly broke the native DNA and the breakage was greatly increased by copper.

A centrifugal fractionation of alkaline solution disclosed, however, that a single strand-scission of native nucleic acid, as is obvious from Fig. 4, can be brought about by 5×10^{-3} M dehydroascorbate alone.

The denatured DNA is, as indicated in Fig. 5, more easily spilt by dehydroascorbate than native one and the splitting considerably intensified in the presence of Cu-salt.

Decomposition of Pyrimidine Nucleide Cluster by Dehydroascorbic Acid. That apurinic acid could be decomposed by the combined action of dehydroascorbate and copper is evident from Fig. 6. The curve suggested that the molecular weight of the product is pretty low.

Since it was found in preliminary tests that the product can pass through Visking-cellulose tube, its separation was performed using this membrane. As is clear from Fig. 7, deoxy-cytidylic acid only was detected by chromatography. Apyrimidinic acid, however, was not disintegrated by dehydroascorbate + Cu.

Finally, as can be seen from Fig. 8, it was observed in the column-chromatographic analysis that dehydroascorbic acid in cooperation with copper decomposes pyrimidine tetra- and pentanucleotides. The decomposition of pyrimidine mono-, di- or trinucleotide did not occur in this case. In the control, we confirmed that the disintegration of purine nucleotide is not caused by the same treatment.

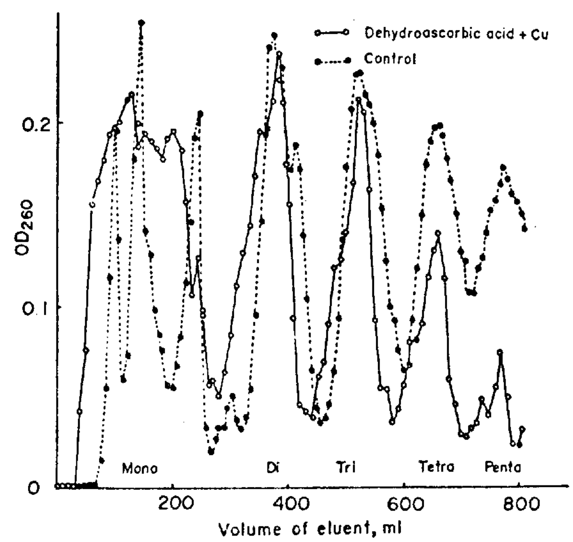

Fig. 8. Column-chromatography pattern of pyrimidine nucleotide after treating with dehydroascorbate + Cu

Discussion

In the preceding study, we have proved that metabolic cancer repression can be accomplished by the action of Dopa, dopamine, noradrenalin and adrenalin

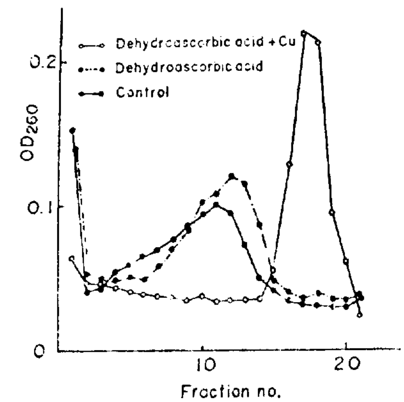

Fig. 3. Sedimentation pattern of native DNA at pH 7 after treating with dehydroascorbate + Cu

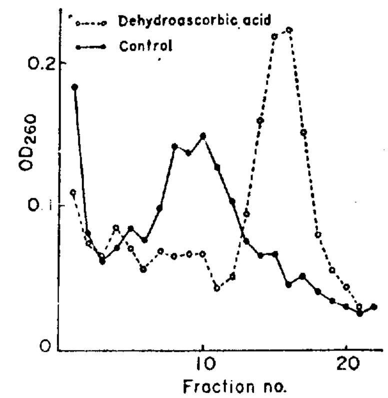

Fig. 4. Sedimentation pattern of native DNA at pH 12.8 after treating with dehydroascorbate

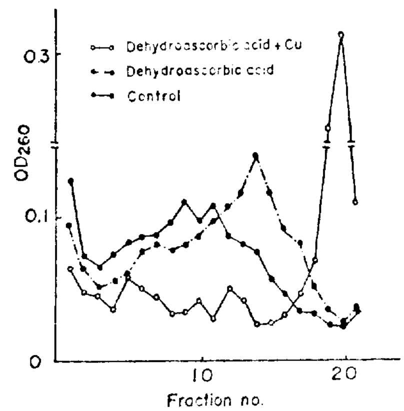

Fig. 5. Sedimentation pattern of denatured DNA after treating with dehydroascorbate + Cu

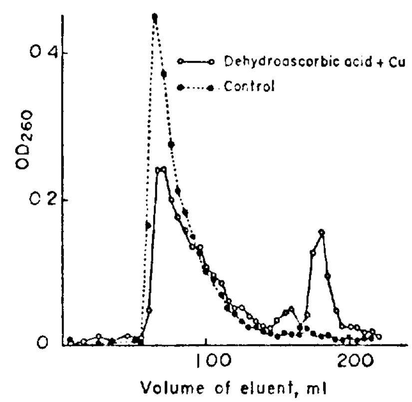

Fig. 6. Gel-filtration pattern of apurinic acid after treating with dehydroascorbate + Cu

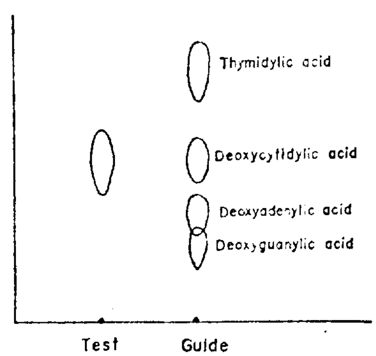

Fig. 7. Paper-chromatography pattern of decomposition product of apurinic acid by dehydroascorbate + Cu

(Yamafuji *et al.*, 1970). Our present investigation may represent another example of biological carcinostasis, starting from vitamin C.

Ascorbic acid is transformed to dehydroascorbic and 2.3-diketogulonic acids in tissues. The above-mentioned experiments have demonstrated that these three enediols are able to break native and dentured DNA. They further produce single strand scission in double helix-form. Recently it has been found that RNA-polymerase is markedly affected by DNA-breakages (Vogt, 1969; Yamafuji *et al.*, 1971–1). The tumour suppression by ascorbate-derivatives is to be attributed either to the abnormality of DNA-replication or to that of mRNA-synthesis.

It has also been here discovered that the product of apurinic acid decomposition by dehydroascorbate + Cu is deoxy-cytidylic acid, and that only the oligo-form of pyrimidine nucleotides suffers disintegration by this reagent. As the distribution of pyrimidine-rich sequences has been reported already (e.g. Mushinsky and Spencer, 1970), we can now conclude that ascorbate-metabolites cleaves the position of such a cluster, according to our schema (Murakami and Yamafuji, 1970). It is thus assumed that the special cleavage of pyrimidine oligonucleotide-sites of structural distortion brings about a genetical alteration, in connection with the deviation of RNA-polymerase activity. Actually we have corroborated that some enediol compounds regulate the gene action (Yamafuji *et al.*, 1971–3), which leads to the formulation of Yamafuji effect causing cellular differentiation and anomalization (Yamafuji *et al.*, 1971–2).

References

Habermann, V.: The degradation of apyrimidinic deoxyribonucleic acid in alkali. A method for the isolation of purine nucleotide sequences from deoxyribonucleic acid. Biochim. biophys. Acta (Amst.) **55**, 999 (1962).

Kagawa, H., Takiguchi, H.: Enzymatic study on the decomposition of ascorbic acid. Symp. Enzyme Chem. Japan **16**, 107 (1962).

Murakami, H., Yamafuji, K.: Mode of action of some catecholamines and sugar oximes on deoxyribonucleic acid. Enzymologia **38**, 337 (1970).

Mushynski, W. E., Spencer, J. H.: Nucleotide clusters in deoxyribonucleic acids. V. The pyrimidine oligonucleotides of strands *r* and *l* of bacteriophage T7 DNA. J. molec. Biol. **52**, 91 (1970).

Nakamura, Y., Yamafuji, K.: Antitumour activities of oxidized products of ascorbic acid. Sci. Bull. Fac. Agr. Kyushu Univ. **23**, 119 (1968).

Roe, J. H., Mills, M. B., Oesterling, M. J., Damron, C. M.: The determination of diketo-L-gulonic acid, dehydro-L-ascorbic acid and L-ascorbic acid in the same tissue extract by the 2,4-dinitropehnolhydrazine method. J. biol. Chem. **174**, 201 (1948).

Spencer, J. H., Chargaff, E.: Pyrimidine nucleotide sequences in deoxyribonucleic acids. Biochim. biophys. Acta (Amst.) **51**, 209 (1961).

Takemura, S.: Hydrazinolysis of nucleic acid, I. The formation of deoxyriboapyrimidinic acid from herring sperm DNA. Bull. chem. Soc. Japan **32**, 920 (1959).

Tamm, C., Hodes, M. E., Chargaff, E.: The formation of apurinic acid from the deoxyribonucleic acid of calf thymus. J. biol. Chem. **195**, 49 (1952).

Vogt, V.: Breaks in DNA stimulate transcription by core RNA polymerase. Nature (Lond.) **223**, 854 (1969).

... K ... A ... new potentiality of polyhedral

J. Biol. Chem. 235: 165-168, 1960.

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Studies on the Defect in Tyrosine Metabolism in Scorbutic Guinea Pigs

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(Received for publication, September 17, 1959)

Scorbutic guinea pigs and man excrete p-hydroxyphenylpyruvic acid in the urine when given large amounts of tyrosine or phenylalanine (1-5). Since the administration of ascorbic acid abolishes this defect, it has been concluded that ascorbic acid has an important role in maintaining normal tyrosine metabolism. At one time, ascorbic acid was proposed as a specific cofactor for p-hydroxyphenylpyruvic acid oxidase, the enzyme which catalyzes the oxidation of p-hydroxyphenylpyruvic acid to homogentisic acid (6). However, a less specific role for the vitamin in this reaction was suggested by the finding that a number of compounds such as D-ascorbic acid, D-isoascorbic acid, and structurally unrelated compounds such as 2,6-dichlorophenolindophenol and hydroquinone could replace ascorbic acid in the oxidation of tyrosine *in vitro* (7, 8). More recent studies (8-10), have shown that ascorbic acid and the compounds which can replace it act by protecting p-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate. This inhibition is unusual in that it occurs after a lag period, and the initial rate of oxidation of the substrate is unaffected. In the presence of ascorbic acid or the other reducing agents, the initial rate of oxidation of p-hydroxyphenylpyruvic acid is continued (10). As a result of these enzyme studies, it was of interest to determine whether vitamin C protects p-hydroxyphenylpyruvic acid oxidase from inhibition *in vivo* and maintains normal tyrosine metabolism by the same mechanism.

In this paper, data will be presented which show that livers of scorbutic guinea pigs contain the same concentration of p-hydroxyphenylpyruvic acid oxidase activity as normal liver. However, the enzyme in the scorbutic liver is vulnerable to substrate inhibition whereas that in the normal liver is protected by the ascorbic acid present. The administration of p-hydroxyphenylpyruvic acid has little if any effect upon the activity of oxidase in the normal animal, but in the scorbutic animals over one-half of the enzyme is inactive 1 hour after a single injection of p-hydroxyphenylpyruvic acid.

EXPERIMENTAL

Materials—p-Hydroxyphenylpyruvic acid was obtained from H. M. Chemical Company, Ltd., Santa Monica, California. 2,6-Dichlorophenolindophenol and α,α′-dipyridyl were purchased from Eastman Organic Chemicals Department, Eastman Kodak Company. Glutathione, L-tyrosine, and α-ketoglutaric acid were commercial preparations from the Nutritional Biochemical Corporation, Cleveland, Ohio. L-Ascorbic acid was obtained from Merck and Company, Inc. Homogentisic acid was purchased from Cylo Chemical Corporation, Los Angeles, California.

Animals—Male, albino guinea pigs weighing approximately 200 g were placed on a diet of either Purina chow rabbit pellets and cabbage (normal diet) or the vitamin C-free diet prepared as described by Woodruff *et al.* (11).[1] In addition, some animals were given the vitamin C-free diet supplemented with 25 mg of ascorbic acid per day. After 2 weeks, the animals on the vitamin C-free diet developed the typical signs of scurvy; weight loss, swollen joints, and hemorrhages in the knee joints. The animals subjected to an intraperitoneal injection of p-hydroxyphenylpyruvic acid were given 20 mg of the neutralized keto acid per 100 g body weight 1 hour before being sacrificed. In order to be certain that the injection was intraperitoneal, blood samples were taken 15 minutes after the injection and the plasma level of the keto acid determined by measuring it as the enol-borate complex spectrophotometrically (12). In each case the animals receiving p-hydroxyphenylpyruvic acid had a high blood level of the keto acid, in the order of 50 μg per ml of plasma.

Preparation of Liver Homogenate—At the time of sacrifice, 6 g of liver were homogenized with 12 ml of 0.2 M sodium phosphate buffer, pH 6.5, with a Potter-Elvehjem type glass homogenizer at 5°. The homogenate was centrifuged at 10,000 × g for 10 minutes, and the resulting supernatant fraction was used in the experiments described below. Protein was determined by the spectrophotometric method of Warburg and Christian (13). The centrifuged homogenate contained approximately 80 mg of protein per ml in both the normal and scorbutic liver preparations.

Liver Ascorbic Acid Determination—Two grams of liver were homogenized with 25 ml of 4 per cent trichloroacetic acid at 5°. After centrifugation, suitable aliquots of the deproteinized supernatant fraction were taken for the determination of ascorbic acid according to the method of Roe *et al.* (14).

Enzyme Assay Methods—Tyrosine transaminase activity was estimated manometrically as described previously (15). The homogenate was supplemented with α-ketoglutarate and 2,6-dichlorophenolindophenol in order to insure optimal activity. p-Hydroxyphenylpyruvic acid oxidase was determined spectrophotometrically by following the decrease of the enol-borate complex in the presence of hog kidney tautomerase as developed by Hager *et al.* (16). Homogentisic acid oxidase was determined manometrically as described previously (15).

RESULTS

Reactivation of p-Hydroxyphenylpyruvic Acid Oxidase Inhibited by Excess Substrate—If ascorbic acid protects p-hydroxyphenyl-

[1] Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

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TABLE I

Reactivation of substrate-inhibited p-hydroxyphenylpyruvic acid oxidase with reduced 2,6-dichlorophenolindophenol

The reaction was measured spectrophotometrically by following the disappearance of the enol-borate complex of *p*-hydroxyphenylpyruvate at 340 mμ (10). The cuvettes contained 0.42 M boric acid 0.17 M sodium phosphate buffer, pH 6.2, 15 μmoles of neutralized glutathione, 0.2 ml of keto-enol tautomerase, and 1.0 μmole of *p*-hydroxyphenylpyruvic acid; after equilibrium 0.1 ml of liver homogenate prepared from a scorbutic guinea pig was added to start the reaction. Total volume was 3.0 ml.

Conditions	Initial velocity
	O.D. × 10³/min
Rate in absence of reduced dye	85
Rate in presence of reduced dye*	88
Rate during substrate inhibition†	35
Rate after addition of reduced dye†	65

* Cuvettes contained 100 μg of 2,6-dichlorophenolindophenol added before the substrate and after glutathione.

† Within 8 minutes the reaction slowed down when dye was omitted and at this time 100 μg of dye were added to duplicate cuvettes and reactivation of inhibited enzyme determined during the next 4 minutes.

pyruvic acid oxidase from inhibition by its substrate *in vivo*, one would expect to find inhibited enzyme in the liver of scorbutic animals after the administration of *p*-hydroxyphenylpyruvic acid. A means of detecting inhibited enzyme was suggested by studies *in vitro* in which it was found that reduced 2,6-dichlorophenolindophenol not only can prevent inhibition by excess substrate but also has the property of reactivating enzyme previously inhibited by substrate (10, 16) or by prolonged storage (17). This effect of 2,6-dichlorophenolindophenol is illustrated in Table 1. If all of the enzyme is present in an active form, the initial rate of substrate disappearance is the same whether or not 2,6-dichlorophenolindophenol is present. However, if part of the enzyme has been previously inactivated by excess substrate, reduced dye reactivates the inhibited portion, and therefore the initial rate will be greater when 2,6-dichlorophenolindophenol is present. It is evident that a large fraction of the activity lost by substrate inhibition in the liver homogenate from a scorbutic guinea pig can be restored by 2,6-dichlorophenolindophenol (Table 1). Therefore, a comparison of the initial rate of substrate disappearance measured both with and without the dye permits an estimation of the relative amount of inactive *p*-hydroxyphenylpyruvic acid oxidase.

Effect of Intraperitoneal Injection of p-Hydroxyphenylpyruvic Acid in Normal and Scorbutic Animals—Eighteen guinea pigs were placed on a pellet and cabbage diet and the same number of animals were given the ascorbic acid deficient diet. After 19 days, the two groups were divided and half of each group received an intraperitoneal injection of *p*-hydroxyphenylpyruvic acid 1 hour before sacrifice. The *p*-hydroxyphenylpyruvic acid oxidase activity of liver homogenates prepared from these animals was then determined spectrophotometrically, with and without 2,6-dichlorophenolindophenol, and typical results in the scorbutic group are shown in Figs. 1 and 2. As can be observed, the initial rate of *p*-hydroxyphenylpyruvic acid oxidation was the same whether or not 2,6-dichlorophenolindophenol was present in the homogenate of an untreated animal (Fig. 1). However, in the homogenate from an animal which had received *p*-hydroxyphen-

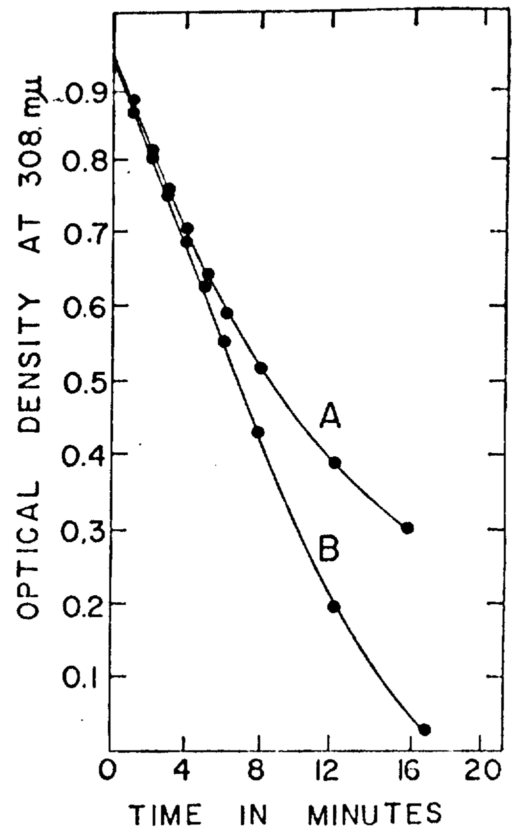

FIG. 1. *p*-Hydroxyphenylpyruvic acid oxidase activity in scorbutic liver homogenate from a guinea pig not treated by injection with *p*-hydroxyphenylpyruvic acid. The reaction was measured spectrophotometrically. The cuvettes contained 0.42 M boric acid-0.17 M sodium phosphate buffer, pH 6.2, 15 μmoles of glutathione, 0.2 ml of keto-enol tautomerase, and 0.25 μmole of *p*-hydroxyphenylpyruvic acid; after equilibrium 0.1 ml of a 33 per cent homogenate was added to start the reaction. Total volume was 3.0 ml. *Curve A*, no reduced 2,6-dichlorophenolindophenol present, *Curve B*, 0.3 μmole of 2,6-dichlorophenolindophenol added after glutathione and before the substrate.

ylpyruvic acid (Fig. 2), the initial rate was at least three times as great in the presence of 2,6-dichlorophenolindophenol. Ascorbic acid was less effective in reactivating the inhibited oxidase than the dye. It should be noted that in the presence of 2,6-dichlorophenolindophenol, the activity of the oxidase was about the same in both homogenate samples, suggesting that approximately two-thirds of the oxidase had been inactivated in the scorbutic animals which had received the injection.

Since large amounts of the substrate had been injected, it was of interest to determine whether any residual *p*-hydroxyphenylpyruvic acid remained in the liver of the treated animals at the time of sacrifice. Analysis of the liver homogenates showed that they contained no detectable amount of the injected compound. Prolonged dialysis of the homogenate did not reactivate it unless the dye or ascorbic acid were added. Thus, the homogenate from the scorbutic animal treated with *p*-hydroxyphenylpyruvic acid behaved in all respects like a preparation of *p*-hydroxyphenylpyruvic acid oxidase previously inhibited by excess substrate (10).

The activity of *p*-hydroxyphenylpyruvic acid oxidase in the normal animals was found to be essentially the same whether or not *p*-hydroxyphenylpyruvic acid had been administered, and reduced 2,6-dichlorophenolindophenol had no effect on the activity of these preparations (Table II). Similar results were obtained in six animals on a vitamin C-deficient diet which was supplemented with 25 mg of ascorbic acid per day. A comparison of the results in the normal and scorbutic groups indicates that the activity of *p*-hydroxyphenylpyruvic acid oxidase in the scorbutic animals which had received *p*-hydroxyphenylpyruvic acid was diminished, and that reduced dye had the capacity to reactivate the inhibited enzyme to the same level as found in normal animals (Table II). It should be noted that the level of *p*-hydroxyphenylpyruvic acid oxidase in the untreated scorbutic animals was essentially the same as that in the normal group and was not increased by the addition of 2,6-dichlorophenolindophenol. Therefore, it appears that the scorbutic animal can maintain normal tyrosine metabolism and has the same concentration of enzyme as the normal animal unless subjected to the effect of large amounts of *p*-hydroxyphenylpyruvic acid. It also appears that under the above conditions in which the *p*-hydroxyphenylpyruvic acid oxidase is inhibited, tyrosine transaminase and homogentisic acid oxidase are not affected.

DISCUSSION

The data presented indicate that ascorbic acid maintains normal tyrosine metabolism *in vivo* through the same mechanism as has been found *in vitro* (9, 10, 16), that is, by protecting *p*-hydroxyphenylpyruvic acid oxidase from inhibition. It is of interest that inhibited *p*-hydroxyphenylpyruvic acid oxidase was found in only the scorbutic animals given *p*-hydroxyphenylpyruvic acid, and that the untreated scorbutic animals had no inhibited liver enzyme. This finding supports the view that ascorbic acid is not needed to maintain tyrosine oxidation under ordinary circumstances or even in the scorbutic state, but it is required under the particular conditions in which inactivation of *p*-hydroxyphenylpyruvic acid oxidase would otherwise occur. In accord with the above data, the accumulation of sufficient substrate to inhibit *p*-hydroxyphenylpyruvic acid oxidase would be favored by an adaptive increase in the tyrosine transaminase activity. An increase in the activity of this enzyme has been demonstrated to occur in rats subjected to stress conditions (18), and in response to treatment with cortisone (19). Feeding extra tyrosine to rats (20) did not alter the *p*-hydroxyphenylpyruvic acid oxidase activity in the liver, but this result is in keeping with the ability of rats to synthesize ascorbic acid. In contrast, guinea pigs fed extra tyrosine excreted *p*-hydroxyphenylpyruvic acid in the urine (1, 21), and this defect is corrected by vitamin C. Without this vitamin, it would be expected that decreased *p*-hydroxyphenylpyruvic acid oxidase activity would help to perpetuate the metabolic defect once conditions have been established which favor inhibition of the oxidase.

Although the exact manner in which ascorbic acid protects the enzyme from substrate inhibition is not known, the possibility must be considered that the vitamin is acting indirectly through an agent or metabolite to protect the oxidase (22). The effectiveness of reduced 2,6-dichlorophenolindophenol in preventing substrate inhibition *in vitro* is about 700 times that of ascorbic acid on a molar basis, and the effectiveness of the vitamin progressively decreases upon purification of the enzyme (10). Ascorbic acid appears to be much more potent *in vivo* considering its low concentration in the liver than it is in preventing substrate inhibition with purified enzyme. Preliminary experiments with six scorbutic animals given reduced 2,6-dichlorophenolindophenol dye several hours before an injection of *p*-hydroxyphenylpyruvic acid indicate that the dye can prevent inhibition of *p*-hydroxyphenylpyruvic acid oxidase *in vivo*. More detailed appraisal of the antiscorbutic activity of 2,6-dichlorophenolindophenol is now under investigation. These studies, as those with D-ascorbic acid (23) may help to differentiate some of the nonspecific functions of vitamin C from those which require L-ascorbic acid.

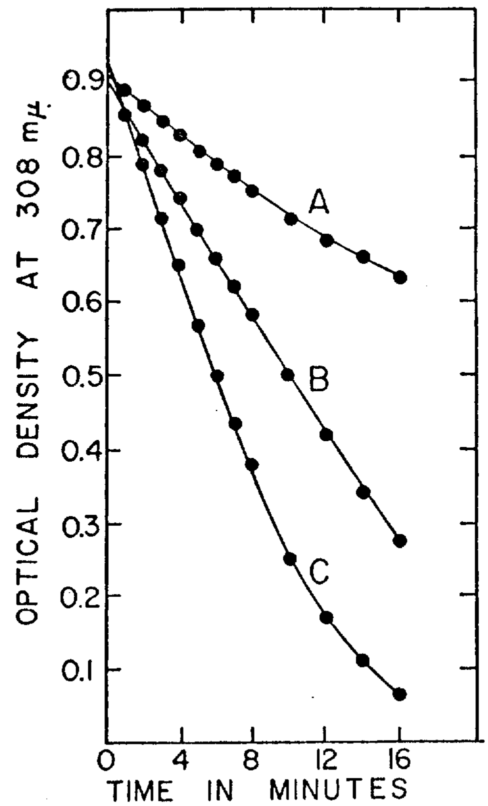

FIG. 2. *p*-Hydroxyphenylpyruvic acid oxidase activity in scorbutic liver homogenate from a guinea pig injected intraperitoneally with *p*-hydroxyphenylpyruvic acid 1 hour before sacrifice. The spectrophotometric assay conditions were as described under Fig. 1. *Curve A*, no ascorbic acid or 2,6-dichlorophenolindophenol present, *Curve B*, 12 μmoles of freshly prepared L-ascorbic acid added after glutathione and before the substrate, *Curve C*, 0.3 μmole of 2,6-dichlorophenolindophenol added after glutathione and before the substrate.

SUMMARY

1. Scorbutic guinea pigs which received parenteral injections of *p*-hydroxyphenylpyruvic acid have over half of their liver *p*-hydroxyphenylpyruvic acid oxidase inhibited 1 hour after the injection. The inactive enzyme can be reactivated *in vitro* by the addition of either ascorbic acid or, more effectively, by the reduced form of 2,6-dichlorophenolindophenol.

2. The activities of liver tyrosine transaminase and homogentisic acid oxidase are not altered by the injection of *p*-hydroxyphenylpyruvic acid in normal or scorbutic guinea pigs.

3. Scorbutic guinea pigs contain the same concentration of *p*-hydroxyphenylpyruvic acid oxidase in the liver as animals on a normal diet.

TABLE II

Effect of an injection of p-hydroxyphenylpyruvic acid on tyrosine transaminase, p-hydroxyphenylpyruvic acid oxidase, and homogentisic acid oxidase in normal and scorbutic guinea pig liver

Assay conditions. *Tyrosine transaminase*—The Warburg flasks contained 1.0 ml of a 33 per cent liver homogenate, 0.2 M sodium phosphate buffer, pH 7.5, 15 μmoles of α-ketoglutarate, 2 μmoles of α,α'-dipyridyl, 20 μmoles of neutralized glutathione, and 100 μg of 2,6-dichlorophenolindophenol dye. The side arm contained 5 μmoles of L-tyrosine in 0.5 ml of the phosphate buffer. Total fluid volume was 2.0 ml. *p-Hydroxyphenylpyruvic acid oxidase*—Assayed as described under Fig. 1. *Homogentisic acid oxidase*—The Warburg flasks contained 0.5 ml or 1.0 ml of a 33% liver homogenate, 0.2 M sodium phosphate buffer, pH 6.5, and 0.2 μmole of ferrous sulfate. The side arm contained 5.0 μmoles of homogentisic acid in 0.5 ml of the phosphate buffer. Total fluid volume was 2.0 ml.

Enzyme assay*	Normal animals†		Scorbutic animals†	
	Untreated (9)‡	Treated (9)	Untreated (8)	Treated (9)
	μmoles of substrate oxidized/hr/g fresh liver§			
Tyrosine transaminase	19.2 ± 3.3	18.1 ± 3.6	25.9 ± 7.0	25.3 ± 4.9
p-Hydroxyphenylpyruvic acid oxidase without reduced dye	32.7 ± 3.2	28.8 ± 4.1	27.0 ± 2.2	12.6 ± 2.9
p-Hydroxyphenylpyruvic acid oxidase with reduced dye	32.3 ± 4.1	27.7 ± 3.8	29.9 ± 4.9	26.1 ± 3.4
Homogentisic acid oxidase	92.7 ± 11.4	100.2 ± 14.8	74.6 ± 18.5	77.4 ± 12.4

* Enzyme assays were carried out at 37° except *p*-hydroxyphenylpyruvic acid oxidase, which was measured at 25° with and without dye (2,6-dichlorophenolindophenol). Enzyme activities are based upon the initial rate of oxidation. Transaminase activity was calculated after the initial lag period.

† Ascorbic acid levels: normal liver, 20.4 ± 5.5 (mean, ± standard error) mg per 100 g wet weight of liver; scorbutic liver, 1.42 ± 0.34.

‡ Numbers in parentheses = number of animals.

§ Standard error of the mean is given. The statistical significance of the difference in *p*-hydroxyphenylpyruvic acid oxidase in the scorbutic animals with and without injection evaluated by the t test gives a P value of <0.001.

4. The ability of ascorbic acid to maintain normal tyrosine metabolism *in vivo* appears to be mediated by its capacity to protect *p*-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate.

REFERENCES

1. SEALOCK, R. R., AND SILBERSTEIN, H. E., *J. Biol. Chem.*, **135**, 251 (1940).
2. SEALOCK, R. R., PERKINSON, J. D., JR., AND BASINSKI, D. H., *J. Biol. Chem.*, **140**, 153 (1941).
3. LEVINE, S. Z., GORDON, H. H., AND MARPLES, E., *J. Clin. Invest.*, **20**, 209 (1941).
4. ROGERS, W. F., AND GARDNER, F. H., *J. Lab. Clin. Med.*, **34**, 1491 (1949).
5. MORRIS, J. E., HARPUR, R. T., AND GOLDBLOOM, A., *J. Clin. Invest.*, **29**, 325 (1950).
6. SEALOCK, R. R., GOODLAND, R. L., SUMERWELL, W. N., AND BRIERLY, J. M., *J. Biol. Chem.*, **196**, 761 (1952).
7. LA DU, B. N., JR., AND GREENBERG, D. M., Science, **117**, 111 (1953).
8. KNOX, W. E., in W. D. MCELROY AND H. B. GLASS (Editors), *A symposium on amino acid metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 836.
9. LA DU, B. N., AND ZANNONI, V. G., *J. Biol. Chem.*, **217**, 777 (1955).
10. ZANNONI, V. G., AND LA DU, B. N., *J. Biol. Chem.*, **234**, 2925 (1959).
11. WOODRUFF, C. W., CHERRINGTON, M. E., STOCKELL, A. K., AND DARBY, W. J., *J. Biol. Chem.*, **178**, 861 (1949).
12. KNOX, W. E., AND PITT, B. M., *J. Biol. Chem.*, **225**, 675 (1957).
13. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **310**, 384 (1941–1942).
14. ROE, J. H., MILLS, M. B., OESTERLING, M. J., AND DAMRON, C. M., *J. Biol. Chem.*, **174**, 201 (1948).
15. LA DU, B. N., ZANNONI, V. G., LASTER, L., AND SEEGMILLER, J. E., *J. Biol. Chem.*, **230**, 251 (1958).
16. HAGER, S. E., GREGERMAN, R. I., AND KNOX, W. E., *J. Biol. Chem.*, **225**, 935 (1957).
17. ROKA, L., KÖNIG, G., AND RÜBNER, H., *Z. physiol. Chem.*, **313**, 87 (1958).
18. MCELROY, O. E., ANDERSON, P. R., AND GRAY, I., *Arch. Biochem. Biophys.*, **76**, 69 (1958).
19. LIN, E. C. C., AND KNOX, W. E., *J. Biol. Chem.*, **233**, 1186 (1958).
20. LIN, E. C. C., AND KNOX, W. E., *Proc. Soc. Exptl. Biol. Med.*, **96**, 501 (1957).
21. PAINTER, H. A., AND ZILVA, S. S., *Biochem. J.*, **41**, 511 (1947).
22. LA DU, B. N., AND ZANNONI, V. G., *J. Biol. Chem.*, **219**, 273 (1956).
23. BURNS, J. J., FULLMER, H. M., AND DAYTON, P. G., *Proc. Soc. Exptl. Biol. Med.*, **101**, 46 (1959).

Biochem Pharmacol 21(10): 1377-1392. Illus. 1972

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Biochemical Pharmacology, Vol. 21, pp. 1377–1392. Pergamon Press, 1972. Printed in Great Britain.

ASCORBIC ACID AND DRUG METABOLISM*

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(*Received* 3 *October* 1971; *accepted* 17 *December* 1971)

Abstract—Studies *in vitro* with liver microsomes isolated from vitamin C deficient guinea pigs have indicated a significant decrease in over-all drug oxidation, as typified by aniline hydroxylation, aminopyrine *N*-demethylation and *p*-nitroanisole *O*-demethylation. Concommitant with decreased over-all drug oxidation, the quantity of cytochrome P-450 and cytochrome b_5, and the activity of NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase also decreased significantly. It was not until the quantity of liver microsomal ascorbic acid had reached 30 per cent of normal values (11 μg/g wet weight compared to 3·5 μg/g wet weight) that a marked decrease in over-all drug oxidation activity, as well as the level and activity of individual electron transport components, occurred. In addition, the decrease in drug enzyme activities was not due to a caloric deficiency in the vitamin C deficient guinea pigs, since studies with fasted animals indicated normal or greater than normal drug enzyme activities. K_m studies with microsomes isolated from normal and vitamin C deficient guinea pigs did not indicate a correlation in the apparent affinity of drug substrates such as aniline, aminopyrine, and *p*-nitroanisole with decreased microsomal enzyme activities in the vitamin C deficient guinea pigs. The K_m value of aniline hydroxylase for aniline was approximately four times higher in normal guinea pig microsomes while the K_m value of *N*-demethylase for aminopyrine and *O*-demethylase for *p*-nitroanisole was in the order of four times higher in the vitamin C deficient guinea pig microsomes. Studies concerned with aniline–cytochrome P-450 binding spectra indicated an atypical altered spectrum with microsomes isolated from vitamin C deficient animals. The usual trough of the spectrum shifted from 390 to 405 nm and the usual peak of the spectrum shifted from 430 to 440 nm. Also, the intensity of the trough and peak was at least 50 per cent lower than normal. Administration *in vivo* of ascorbic acid to vitamin C deficient animals was followed by reversal of over-all drug oxidation activities, quantity of cytochrome P-450, NADPH cytochrome P-450 reductase activity and altered aniline–cytochrome P-450 binding, but these changes required at least 6 days of treatment to return to normal even though normal levels of liver ascorbic acid were established within 3 days. Phenobarbital induction studies indicated that the microsomal protein-synthesizing system responds to such treatment in vitamin C deficient guinea pigs, and the increase in drug enzyme activities and the level and activity of electron transport components are equal to, if not greater than, those observed in normal animals. Alternative pathways involving an ascorbic acid dependent NADH oxidase system, which has been shown to be capable of metabolizing drugs in the absence of a microsomal NADPH P-450 reductase system, have been considered. The significance of vitamin C deficiency in human drug therapy has been discussed.

It HAS been known for some time that the activity of liver microsomal drug-metabolizing enzymes is influenced by many factors such as age, sex, strain and species, as well as the nutritional state of the animal. In addition, induction of drug enzyme activities has been shown to occur by foreign compounds including drug substrates,

* This study was supported by Grant GM 17184 from the National Institutes of Health, United States Public Health Service and in part by Grant 23007 from Hoffmann-La Roche, Inc.

insecticides, food additives and polycyclic carcinogenic hydrocarbons such as 3-methylcholanthrene.[1,2]

In regard to nutrition, previous studies *in vivo* in vitamin C deficient guinea pigs have suggested a role for ascorbic acid in drug metabolism. Early investigations by Richards *et al.*[3] and Richards[4] indicated that vitamin C deficient guinea pigs were more sensitive to pentobarbital and procaine than animals not deprived of the vitamin. In 1954, Axelrod *et al.*[5] showed a significant increase in the plasma biological half-life of acetanilide, antipyrine and aniline in guinea pigs depleted of ascorbic acid. These studies *in vivo* indicated that the rate of hydroxylation of these compounds was markedly reduced in vitamin C deficiency. In addition, Conney *et al.*[6] have shown that vitamin C deficient guinea pigs, with no obvious signs of scurvy, had a marked sensitivity to the muscle relaxant zoxazolamine. The increased duration of this drug *in vivo* could be explained by a concomitant decrease in its oxidation *in vitro* in vitamin C deficient guinea pig liver microsomes. In 1968, Degkwitz *et al.*[7] reported that scorbutic guinea pigs have a marked reduction in the hydroxylation of coumarin. Recently Kato *et al.*[8] have shown that the hydroxylation of aniline, hexobarbital and zoxazolamine decreased in guinea pigs on a vitamin C free diet for 12 days. However, the *N*-demethylation of aminopyrine, diphenhydramine and meperidine was not affected, nor were there changes in the *O*-demethylation of *p*-nitroanisole and the reduction of *p*-nitrobenzoic acid and *p*-dimethylaminoazobenzene. In addition, these authors found no significant decrease in individual liver microsomal electron transport components, such as cytochrome P-450.

In general, the previous studies, which have been in the main investigations *in vivo*, have shown decreased metabolism of a variety of pharmacological agents in vitamin C deficient animals, although some of these reports are conflicting as to the type of over-all oxidation reactions affected. In addition, very little information is available in regard to the underlying biochemical basis of decreased drug metabolism in vitamin C deficiency. The data presented in this paper are concerned with the type of over-all drug oxidation reactions, such as *N*-demethylation, *O*-demethylation and hydroxylation, affected by ascorbic acid deprivation for various lengths of time as well as the reversal of the impairment in deficient animals. In addition, data are presented on the level and activity of various liver microsomal electron transport components in normal guinea pigs and guinea pigs maintained on a vitamin C deficient diet for 10 and 21 days. Kinetic studies with microsomes isolated from normal and vitamin C deficient guinea pigs were done to determine the apparent Michaelis–Menten affinity constants of various drug enzymes for substrates and cofactors. Studies on the binding characteristics of cytochrome P-450 and drug substrate in normal and vitamin C deficient guinea pig microsomes are given. Furthermore, experiments using phenobarbital to induce the liver microsomal drug-metabolizing system were carried out in normal and vitamin C deficient guinea pigs to examine the effect of vitamin C deficiency on the induction of microsomal protein, electron transport components, and over-all drug oxidation.

MATERIALS AND METHODS

Aniline and *p*-nitroanisole were obtained from Fisher Scientific Company. Aminopyrine was obtained from K & K Laboratories, Inc. Glucose 6-phosphate, NADP, NADPH, cytochrome *c*, ascorbic acid deficient diet (guinea pig), and L-ascorbic acid were obtained from Nutritional Biochemical Corp. Glucose-6-PO_4 dehydrogenase (Grade II; 0·9 units/mg) was obtained from Sigma Chemical Company. Carbon monoxide was obtained from Matheson Gas Products. Phenobarbital (sodium) was obtained from Amend Drug & Chemical Company. Ascorbyl palmitate was obtained from Hoffmann-La Roche, Inc.

Vitamin C deficient guinea pigs. Groups of male albino guinea pigs (200–250 g) were placed on a vitamin C free diet for either 10 days or 21 days. The animals which were on the diet for 10 days showed no significant weight loss, had no joint hemorrhages, and their liver ascorbic acid level was approximately 6 mg/100 g. The animals on the diet for 21 days also showed no appreciable weight loss and on sacrifice had slight leg joint hemorrhages; their liver vitamin C level was 2·5 mg/100 g.

Groups of normal guinea pigs (200–250 g) consisted of animals which were fed a regular Chow diet or animals on a vitamin C deficient diet supplemented with 50 mg of ascorbic acid/day in their drinking water. There was no appreciable difference in these two groups of animals in that their average weight was 260–280 g and their liver ascorbic acid level was in the order of 20 mg/100 g. In the experiments reported, the control animals were placed on a vitamin C diet supplemented with 50 mg of ascorbic acid/day in their drinking water.

Fasted guinea pigs. Male albino guinea pigs (200–250 g) were fasted for 3–4 days. They received 50 mg of ascorbic acid/day in their drinking water. At the end of the fourth day the animals had lost 20–25 per cent of their body weight and their liver ascorbic acid level was approximately 15 mg/100 g.

Phenobarbital induction. Groups of normal and vitamin C deficient (21 days) male albino guinea pigs were given sodium phenobarbital in their drinking water which contained 1 mg of sodium phenobarbital/ml for 4 days. The guinea pigs drank on the average of 30–40 ml/day. There was no significant difference in the water consumption in the two groups. Preliminary experiments with normal and vitamin C deficient animals indicated that maximal induction occurred between 3·5 and 4 days of phenobarbital administration.

Preparation of guinea pig liver microsomes. Guinea pigs were decapitated, exsanguinated, and their livers were quickly removed and placed on ice. All following procedures were performed at 4°. Homogenates (20%, w/v) were prepared with 1·15% KCl in 0·002 M tris buffer, pH 7·4, with a Potter–Elvehjem homogenizer. The crude homogenate was spun at 15,000 *g* for 15 min. The 15,000 *g* supernatant fraction containing the microsomes was spun at 100,000 *g* for 60 min at 2°. The microsomal pellet was suspended to one-half of the 15,000 *g* supernatant volume with 1·15% KCl in 0·002 M tris, pH 7·4, and the protein concentration was in the order of 8–10 mg/ml. All enzyme assays were performed on freshly prepared microsomes.

p-Nitroanisole O-demethylase activity. Microsomal *p*-nitroanisole *O*-demethylase activity was determined by measuring the product of the reaction, *p*-nitrophenol, at 415 nm as previously described.[9,10] *O*-demethylase activity was calculated from the initial rate of the reaction which was linear with time for at least 15 min. Under the conditions of the assay, 1·0 μg of product, *p*-nitrophenol, gives an optical density reading of 0·082 at 415 nm.

The specific activity of *O*-demethylase is expressed as micromoles of *p*-nitrophenol formed per hour per 100 mg of microsomal protein at 27°.

Aminopyrine N-demethylase activity. Microsomal aminopyrine *N*-demethylase activity was determined by measuring the formaldehyde formed during the demethylation of aminopyrine to 4-aminoantipyrine. The liberated formaldehyde is measured by the colorimetric procedure of Nash[11] which is based on the Hantzsch reaction. The rate of the reaction was linear with time for at least 60 min and proportional to enzyme concentration. Under the assay conditions, the formation of 1 μg of HCHO gives an optical density reading of 0·088 at 412 nm.

The specific activity of *N*-demethylase activity is defined as micromoles of HCHO formed per hour per 100 mg microsomal protein at 27°.

Aniline hydroxylase activity. Microsomal aniline hydroxylase activity was determined by measuring the product, *p*-aminophenol, after formation of a complex with phenol reagent at 660 nm according to the method of Brodie and Axelrod.[12] The rate of the reaction was linear with time for at least 60 min and proportional to enzyme concentration. Under the assay condition, the formation of 1 μg of *p*-aminophenol gives an optical density reading of 0·100.

The specific activity of aniline hydroxylase activity is expressed as micromoles of *p*-aminophenol formed per hour per 100 mg microsomal protein at 27°.

NADPH-cytochrome c *reductase activity.* Microsomal NADPH-cytochrome *c* reductase activity was based on the absorbance of reduced cytochrome *c* at 550 nm according to the method of Williams and Kamin.[13] The reaction was followed in a Gilford 2000 recording spectrophotometer, and the rate of the reaction was linear with time for at least 20 min.

Under the conditions of the assay the molar extinction coefficient of reduced cytochrome *c* is 6·37 $mM^{-1}cm^{-1}$.[13] The specific activity of NADPH-cytochrome *c* reductase activity is expressed as micromoles of cytochrome *c* reduced per hour per 100 mg microsomal protein at 27°.

Cytochrome b_5. The determination of the quantity of microsomal cytochrome b_5 is based on the method of Omura and Sato.[14] The amount of cytochrome b_5 present in the microsomes is measured after its reduction with sodium dithionite at 426 nm.

Under the conditions of the assay, the molar extinction coefficient of reduced cytochrome b_5 is 171 $mM^{-1}cm^{-1}$.[14] The specific activity of cytochrome b_5 is expressed as micromoles of cytochrome b_5 per 100 mg liver microsomal protein.

Cytochrome P-450. The quantity of microsomal cytochrome P-450 was determined by the method of Omura and Sato.[15] Cytochrome P-450 is reduced with sodium dithionite and complexed with CO; the latter complex absorbs light at 450 nm. The amount of reduced cytochrome P-450 was proportional to the amount of microsomal protein present. Under the conditions of the assay, the apparent molar extinction coefficient of the reduced P-450–CO complex is 91 $mM^{-1}cm^{-1}$.

The specific activity of cytochrome P-450 is expressed as micromoles of cytochrome P-450 per 100 mg of microsomal protein.

NADPH cytochrome P-450 reductase. The determination of microsomal NADPH cytochrome P-450 reductase activity was based on the formation of reduced cytochrome P-450–CO by NADPH. The reaction was followed in a Gilford 2000 recording spectrophotometer at 15°. A 6·0-ml aliquot of microsomes (8 mg protein/ml) was placed in a Erlenmeyer flask, capped and placed in an ice bath. Carbon monoxide (previously passed through a solution of 0·5% sodium dithionite in 0·10 N NaOH) was bubbled through the microsomal suspension with a 20-gauge needle for 5 min. After this time, 3·0 ml of the microsomal suspension was transferred with a syringe to a rubber capped cuvette (10 mm light path) which had been previously evacuated for 3 min. The sealed cuvette was placed in the spectrophotometer, and the absorbance due to the microsomes was offset to 0 absorbance at 450 nm. Control readings prior to the addition of NADPH were recorded for 10–15 sec. The reaction was initiated by the addition of 0·05 ml of NADPH (final concentration, $8 \cdot 30 \times 10^{-5}$ M) with a syringe and mixed with the microsomal suspension (4–6 sec).

The activity was calculated based on an early rate of 4 sec extrapolated to zero time. The 4-sec rate was used since during this time the velocity of the reaction was, in each case, linear with time (2–3 assays/microsomal preparation) and proportional to enzyme concentration. The rate of the reaction was followed for at least 40 sec and under the assay conditions was found to be linear during this time. In all assays, however, 4-sec rates were used since comparisons could be made with reaction rates at 27°. At this temperature, reaction rates were linear with time for 4 sec, but not thereafter; after 4 sec a slower rate occurred similar to those reported.[16–18] The calculated Q_{10} of the reaction, using 4-sec rates, was 1·91.

Specific activity is defined as micromoles of cytochrome P-450 reduced per hour per 100 mg liver microsomal protein at 27°, using a molar extinction coefficient of 91·0 $mM^{-1}cm^{-1}$.

Aniline-cytochrome P-450 binding spectrum (type II). Aniline (5 mM) was added to a microsomal suspension (2·0 mg protein/ml of 0·05 M tris, pH 7·5). The experimental cuvette (aniline-treated) was scanned from 360 to 500 nm at 5-nm intervals in a Gilford 2000 spectrophotometer. The absorbance at each wavelength minus the absorbance at 500 nm was recorded. The usual type II spectrum was obtained with a trough at 390 nm and a peak at 430 nm.[19] The intensity of the trough and peak was proportional to the concentration of cytochrome P-450 present since aniline was added in excess.

Guinea pig liver ascorbic acid determination. Ascorbic acid was determined in 15,000 *g* supernatant fractions and microsomes by a micro modification of the phenylhydrazine method of Roe and Kuether.[20] Proportional reduction of all reactants were made to give a total fluid volume of 0·92 ml. Under the conditions of the method, 1·0 μg of the phenylhydrazine derivative of dehydroascorbic acid gave an O.D. reading of 0·090 at 540 nm.

Protein was determined with the method of Lowry *et al.*[21] Crystalline bovine serum albumin, Fraction V, was used as a standard.

RESULTS

Drug metabolism in normal and vitamin C deficient guinea pigs. Male albino guinea pigs (200–250 g) were maintained on a vitamin C deficient diet for 10 and 21 days. Normal animals were maintained on a vitamin C deficient diet supplemented with 50 mg of ascorbic acid in their drinking water. All animals were selected from a single group. The activity of over-all drug oxidation reactions as typified by aniline hydroxylation, aminopyrine *N*-demethylation, and *p*-nitroanisole *O*-demethylation and the level and activity of liver microsomal electron transport components such as cytochrome P-450, cytochrome b_5, NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase were determined (Table 1). As can be observed, there was no significant decrease in over-all drug oxidation activity or in the level and activity of microsomal electron transport components in the guinea pigs maintained on a vitamin C deficient diet for 10 days. After 10 days on the deficient diet, the amount of ascorbic acid in the liver was approximately 30 per cent of normal based on the 15,000 g supernatant fraction (194 vs. 62 μg/g wet weight) and 50 per cent of normal based on the microsomal fraction (11 vs. 6 μg/g wet weight). In contrast to these results, guinea pigs that were on the vitamin C deficient diet for 21 days showed a marked decrease in both over-all drug hydroxylation activity and electron transport components. Aniline hydroxylase activity decreased 50 per cent; aminopyrine N-demethylase activity decreased 56 per cent; and p-nitroanisole O-demethylase activity decreased 66 per cent. The amount of cytochrome P-450 decreased 40 per cent, and the activity of NADPH cytochrome P-450 reductase decreased at least 85 per cent: not as significantly, however, NADPH cytochrome c reductase activity and the level of cytochrome b_5 decreased 33 per cent. It is important to point out that, concomitant with the decrease in over-all drug oxidation activity and microsomal electron transport components, there was a further decrease in the amount of ascorbic acid in the 15,000 g supernatant fraction (60 per cent) and microsomal fraction (42 per cent) in the guinea pigs maintained on the vitamin C deficient diet for 21 days as compared to guinea pigs on the diet for 10 days.

TABLE 1. EFFECT OF VITAMIN C DEFICIENCY (10 AND 21 days) ON DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN GUINEA PIG LIVER MICROSOMES

	Activity*			
	Normal	Vitamin C deficient (10 days)	Vitamin C deficient (21 days)	Decrease (%)
Aniline hydroxylase	1·6 ± 0·2 P < 0·001	1·3 ± 0·1	0·8 ± 0·2 P < 0·001	50
Aminopyrine N-demethylase	3·9 ± 0·1 P < 0·001	3·3 ± 0·4	1·7 ± 0·3 P < 0·001	56
p-Nitroanisole O-demethylase	3·2 ± 0·4 P < 0·001	3·0 ± 0·2	1·1 ± 0·2 P < 0·001	66
Cytochrome P-450	0·05 ± 0·01 P < 0·01	0·05 ± 0·001	0·03 ± 0·003 P < 0·01	40
NADPH cytochrome P-450 reductase	0·80 ± 0·2	0·87 ± 0·33	< 0·10†	85
NADPH cytochrome c reductase	124 ± 21 P < 0·05	167 ± 20	83 ± 11 P < 0·05	33
Cytochrome b_5	0·03 ± 0·004 P > 0·05	0·03 ± 0·003	0·02 ± 0·006 P > 0·05	33
Liver ascorbic acid				
Supernatant fraction 15,000 g (μg/g wet weight)	194 ± 29	62 ± 15	25 ± 15	
Microsomal fraction (μg/g wet weight)	11 ± 3·8	6 ± 0·9	3·5 ± 2·0	

* Assay conditions and units of activity are given in Materials and Methods.
† This value is a lower limit of detection. Mean ± S.E. of 10 animals per group.

It should be mentioned that experiments *in vitro* in which attempts were made to restore the activity of aniline hydroxylase, aminopyrine N-demethylase, and p-nitroanisole O-demethylase in vitamin C deficient guinea pig microsomes were unsuccessful. Neither the simultaneous addition of ascorbic acid ($2{\cdot}3 \times 10^{-3}$ M) and

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substrate, nor the pretreatment of the vitamin C deficient guinea pig microsomes with the vitamin had any effect in restoring over-all drug oxidation activity.

Although the animals on the vitamin C deficient diet for 21 days did not have appreciable weight loss (at most 5 per cent), it was important to be certain that the observed effects on drug metabolism were not due to a diminished caloric intake by these animals, since it is known that drug metabolism is influenced by the nutritional state of the animal.[22]

Guinea pigs were deprived of food for 3 days and supplemented with 50 mg of ascorbic acid/day in their drinking water. During this time the animals lost 20–25 per cent of their body weight. At the end of 3 days the animals were sacrificed, and overall drug hydroxylation activities and the activity and level of various electron transport components were determined (Table 2). In contrast to the animals maintained on a vitamin C deficient diet, these animals had, in general, an increase in drug metabolism. Aniline hydroxylase and *p*-nitroanisole *O*-demethylase activities increased approximately 2-fold in the starved animals as compared to normal while aminopyrine *N*-demethylase activity was not significantly altered. In addition, there was an increase in the activities of NADPH cytochrome P-450 reductase (7-fold) and NADPH cytochrome *c* reductase (1·6-fold) in the starved guinea pig. It should be mentioned that the quantity of liver ascorbic acid in the 15,000 *g* supernatant fraction and microsomal fraction was in the same order of magnitude in both groups.

TABLE 2. EFFECT OF VITAMIN C DEFICIENCY (21 days) AND STARVATION (3 days) ON THE ACTIVITY OF DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN GUINEA PIG LIVER MICROSOMES

	Activity*		
	Normal†	Starved‡	Vitamin C deficient†
Aniline hydroxylase	1·6 ± 0·2	3·2 ± 0·5	0·8 ± 0·2
Aminopyrine *N*-demethylase	3·9 ± 0·1	4·8 ± 1·2	1·7 ± 0·3
p-Nitroanisole *O*-demethylase	3·2 ± 0·4	6·2 ± 0·2	1·1 ± 0·2
Cytochrome P-450	0·05 ± 0·01	0·07 ± 0·017	0·03 ± 0·003
NADPH cytochrome P-450 reductase	0·8 ± 0·2	5·4 ± 1·95	< 0·10
NADPH cytochrome *c* reductase	124 ± 21	205 ± 25	83 ± 11
Cytochrome b_5	0·03 ± 0·004	0·03 ± 0·012	0·02 ± 0·006
Liver ascorbic acid			
Supernatant fraction 15,000 *g* (μg/g wet weight)	194 ± 29	144 ± 24	25 ± 15
Microsomal fraction (μg/g wet weight)	11 ± 3·8	13 ± 2·2	3·5 ± 2·0

* Assay conditions and units of activity are given in Materials and Methods.
† Mean ± S.E. of 10 animals per group.
‡ Mean ± S.E. of 8 animals per group.

K_m *determination in liver microsomes from normal and vitamin C deficient guinea pigs.* In general, there was no apparent correlation in the K_m values for various substrates (Table 3) and decreased drug metabolism activity in vitamin C deficient guinea pig liver microsomes (Table 1). For example, the K_m value of aniline hydroxylase for aniline was approximately four times higher using normal guinea pig microsomes as compared to vitamin C deficient guinea pig microsomes. In contrast, the K_m

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value of *N*-demethylase for aminopyrine or *O*-demethylase for *p*-nitroanisole was in the order of three to four times higher using vitamin C deficient guinea pig microsomes. Furthermore, the K_m value of NADPH cytochrome *c* reductase for NADPH was in the same order of magnitude with both microsomal preparations. In addition, determination of V_{max} values indicated no significant differences existed in the values between the normal and vitamin C deficient animals. Although some of the decreased activity of aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase in vitamin C deficient guinea pigs may be explained, in part, by a lower affinity of these enzymes for their substrates, the decreased activity in aniline hydroxylase and NADPH cytochrome *c* reductase cannot be due to an alteration in the apparent affinity of these enzymes for their respective substrates.

Investigations from many laboratories have shown that an obligatory initial step in over-all drug metabolism is the binding of drug substrate with microsomal cytochrome P-450.[19,23] A comparison was made of drug substrate binding with microsomes isolated from normal and vitamin C deficient guinea pigs. For these studies a representative type II substrate, aniline was used since this compound gave reproducible and consistent typical type II spectra with normal guinea pig microsomes (minimum absorption at 390 nm, maximum absorption at 430 nm).

TABLE 3. APPARENT MICHAELIS–MENTEN AFFINITY CONSTANTS (K_m) FOR OVER-ALL HYDROXYLATION REACTIONS*

	Normal	Vitamin C deficient (21 days)
Aniline hydroxylase	17.3×10^{-4} M	4.6×10^{-4} M
Aminopyrine *N*-demethylase	1.7×10^{-3} M	4.0×10^{-3} M
p-Nitroanisole *O*-demethylase	4.5×10^{-5} M	15.3×10^{-5} M
NADPH cytochrome *c* reductase	3.5×10^{-6} M	2.8×10^{-6} M

* Assay conditions for aniline hydroxylase, aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase and NADPH cytochrome *c* reductase, were carried out as described in Materials and Methods. Eight concentrations of each substrate were used in the following range of concentrations: aniline: 5.0×10^{-4} M–1.6×10^{-4} M; aminopyrine: 2.7×10^{-3} M–5.0×10^{-4} M; *p*-nitroanisole: 1.8×10^{-3} M–1.8×10^{-4} M; and NADPH; 5.0×10^{-5} M–5.0×10^{-6} M. K_m values shown are derived from a double reciprocal plot of 1/velocity of product formed vs. 1/substrate concentration.

Aniline-cytochrome P-450 binding spectra in normal and vitamin C deficient guinea pig microsomes. The treatment of normal guinea pig microsomes with aniline resulted in a typical type II spectrum with a trough at 390 and a peak at 430 nm. On the other hand, the treatment of vitamin C deficient guinea pig microsomes with aniline resulted in an altered spectrum (Fig. 1). The trough of the atypical spectrum was at 405 instead of 390 nm for the normal spectrum, and the peak of the atypical spectrum was at 440 instead of 430 nm for the normal spectrum. In addition to a shift in the trough and peak, there were marked differences in the absorption intensity in the atypical vitamin C deficient aniline–cytochrome P-450 spectrum. There was an 80 per cent decrease in the peak and a 60 per cent decrease in the trough (Fig. 1). Also, the general shape of the atypical spectrum was less symmetrical than that obtained with normal guinea pig microsomes. The addition of ascorbic acid (2.3×10^{-3} M), either before or after the addition of aniline, to vitamin C deficient guinea pig microsomes had no effect in

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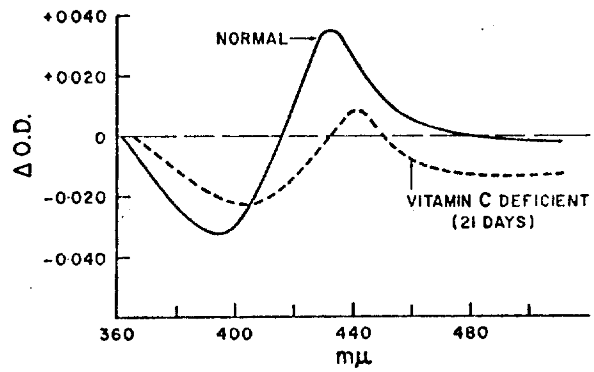

FIG. 1. Aniline–cytochrome P-450 binding spectra (type II) in normal and vitamin C deficient guinea pigs (21 days). Substrate difference spectra were obtained as described in Materials and Methods. The final concentration of aniline was 5 mM and the concentration of microsomal protein was 2 mg/ml. The difference spectra were obtained following the addition of aniline to the microsomes.

restoring the altered spectrum to a typical type II spectrum. It should be mentioned that the microsomes isolated from animals on a vitamin C deficient diet for 10 days, which had normal over-all drug metabolism activity and electron transport components, also showed normal aniline–cytochrome P-450 binding spectra.

Reversal in vivo *of altered drug metabolism activity in vitamin C deficient guinea pigs with ascorbic acid.* Groups of guinea pigs which had been on a vitamin C deficient diet for 21 days were given 50 mg of ascorbic acid/day in their drinking water for 3, 6 and 10 days. At the end of these times the animals were sacrificed, liver microsomes prepared, and the activity of over-all drug oxidation (aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase) and the quantity and activity of individual electron transport components were determined. As can be seen in Fig. 2, aniline hydroxylase activity required 6days to return to normal, *O*-demethyl-

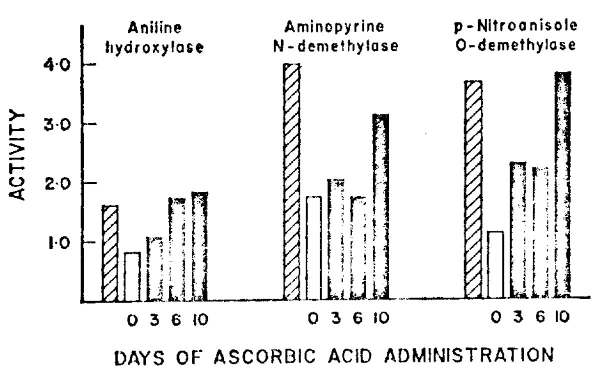

FIG. 2. Reversal of decreased aniline hydroxylase, aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase activities in vitamin C deficient guinea pigs with ascorbic acid. Groups of normal and vitamin C deficient guinea pigs (21 days) were given 50 mg of ascorbic acid in their drinking water for 3, 6 and 10 days as described in Materials and Methods. Aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase were determined as described in Materials and Methods. Enzyme activity equals the micromoles product formed per hour per 100 mg liver microsomal protein at 27°.

ase activity required 10 days, whereas *N*-demethylase activity had reached only 80 per cent of normal after 10 days. The time for reversal was similar for electron transport components in that cytochrome P-450 required 10 days to reach normal levels as did cytochrome b_5 and NADPH cytochrome P-450 reductase (Fig. 3). NADPH

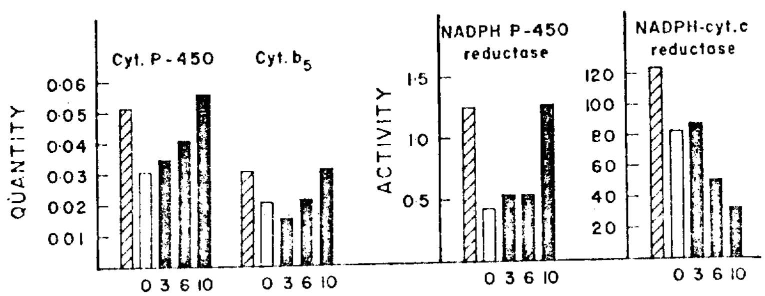

Fig. 3. Reversal of decreased electron transport components in vitamin C deficient guinea pigs with ascorbic acid. Groups of normal and vitamin C deficient guinea pigs (21 days) were given 50 mg of ascorbic acid in their drinking water for 3, 6 and 10 days as described in Materials and Methods. Cytochrome P-450, cytochrome b_5, NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase were determined as described in Materials and Methods. The quantity of cytochrome P-450 equals the micromoles per 100 mg liver microsomal protein. The quantity of cytochrome b_5 equals the micromoles per 100 mg liver microsomal protein. NADPH cytochrome P-450 reductase equals the micromoles of cytochrome P-450 reduced per hour per 100 mg liver microsomal protein at 27°. NADPH cytochrome *c* reductase equals the micromoles of cytochrome *c* reduced per hour per 100 mg liver microsomal protein at 27°.

cytochrome *c* reductase activity did not return to normal even after 10 days and, in fact, was maintained at the low 10-day level for at least 16 days. Of significance is the fact that the quantity of liver ascorbic acid in both the 15,000 *g* liver supernatant fraction and microsomal fraction had returned to normal levels within 3 days of ascorbic acid feeding, while over-all drug oxidation activity and electron transport component levels were still depressed (Table 4).

Table 4. Liver ascorbic acid in vitamin C deficient guinea pigs (21 days) before and after oral administration of ascorbic acid

	Supernatant fraction 15,000 *g* (μg/g wet weight)	Microsomal fraction (μg/g wet weight)
Normal	194	11·0
Vitamin C deficient (21 days)*	25	3·5
Vitamin C deficient + ascorbate (3 days)	258	18·0
Vitamin C deficient + ascorbate (6 days)	241	27·0
Vitamin C deficient + ascorbate (10 days)	227	12·0

* Guinea pigs were on the vitamin C deficient diet for 21 days and groups of 4 were given 50 mg of ascorbic acid/day in their drinking water for the length of time indicated in the table. Ascorbic acid was determined in the liver 15,000 *g* supernatant fraction and microsomal fraction according to the method of Roe and Kuether[20] as described in Materials and Methods.

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The aniline–cytochrome P-450 binding spectra with microsomes isolated from vitamin C deficient guinea pigs which were given ascorbic acid for 3, 6 and 10 days is shown in Fig. 4. As with over-all drug metabolism activities, the typical binding spectrum, that is a trough at 390 and a peak at 430 nm, was not obtained in 3 or 6 days, but was obtained after 10 days of ascorbic acid administration.

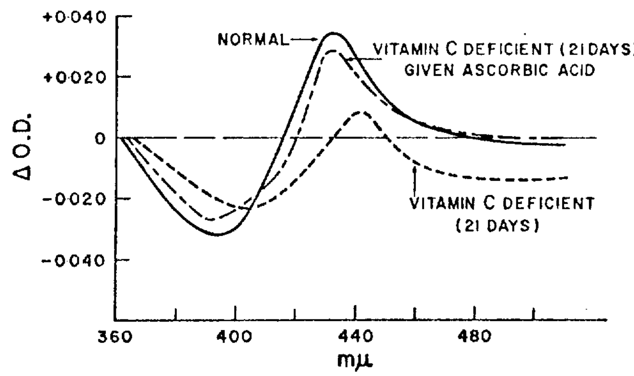

FIG. 4. Reversal of altered aniline–cytochrome P-450 binding spectrum in vitamin C deficient guinea pigs (21 days) given ascorbic acid. Substrate difference spectra were obtained as described in Materials and Methods. The final concentration of aniline was 5 mM, and the concentration of microsomal protein was 2 mg/ml. The difference spectra were obtained following the addition of aniline to the microsomes.

The results with administration *in vivo* of ascorbic acid indicate that although normal levels of ascorbic acid could be achieved within 3 days, there was no reversal of altered drug enzyme activities or atypical binding of drug to cytochrome P-450 until at least 10 days. One possibility is that the time required for reversal in vitamin C deficient guinea pigs depends on the occurrence of newly synthesized microsomal protein. Phenobarbital, a potent drug enzyme inducer which acts by causing a specific increase in microsomal protein, was administered to vitamin C deficient guinea pigs and normal guinea pigs.

Phenobarbital induction in normal and vitamin C deficient guinea pigs. Induction of over-all drug oxidation activities (aniline hydroxylase, aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase) and microsomal electron transport components occurred in both normal and vitamin C deficient guinea pigs (Table 5). The fold increase due to induction (phenobarbital treatment/no treatment) in the vitamin C deficient guinea pigs was in the same order of magnitude, and in some cases greater, than the fold increase in the normal guinea pigs. For example, aniline hydroxylase increased 2·1-fold in the vitamin C deficient animal compared to 1·4-fold in the normal animal, and *p*-nitroanisole *O*-demethylase increased 5·9-fold in the vitamin C deficient animal compared to 3·5-fold in the normal animal. Furthermore, the difference between the level of activity after phenobarbital treatment and basal level (no treatment) in vitamin C deficient and normal guinea pigs was in the same order of magnitude for each enzyme activity. As was found with over-all drug oxidation activities, the individual electron transport components were also induced in vitamin C deficient guinea pigs and the fold increase was equivalent to, or in some cases better than, the normal or vitamin C deficient guinea pigs.

TABLE 5. PHENOBARBITAL INDUCTION OF DRUG ENZYMES AND ELECTRON TRANSPORT COMPONENTS IN NORMAL AND VITAMIN C DEFICIENT GUINEA PIGS

	Activity*					
	Normal†			Vitamin C deficient†		
	No R_x‡	PB R_x§	Fold increase	No R_x‡	PB R_x§	Fold increase
Aniline hydroxylase	1·6 ± 0·2	2·3 ± 0·5	1·4	0·8 ± 0·2	1·7 ± 0·3	2·1
Aminopyrine *N*-demethylase	3·9 ± 0·1	9·6 ± 1·6	2·5	1·7 ± 0·3	7·6 ± 1·7	4·5
p-Nitroanisole *O*-demethylase	3·2 ± 0·4	11·1 ± 2·7	3·5	1·1 ± 0·2	6·5 ± 1·6	5·9
Cytochrome P-450	0·05 ± 0·01	0·11 ± 0·02	2·2	0·03 ± 0·003	0·06 ± 0·01	2·0
NADPH P-450 reductase	0·8 ± 0·2	2·6 ± 0·6	3·3	< 0·10	2·8 ± 1·0	28·0
NADPH cytochrome *c* reductase	124 ± 21	288 ± 49	2·3	83 ± 11	250 ± 49	3·0
Cytochrome b_5	0·03 ± 0·004	0·03 ± 0·006	1·0	0·02 ± 0·006	0·02 ± 0·01	1·0

* Assay conditions, units of activity and phenobarbital treatment are given in Materials and Methods.

† Liver ascorbic acid: normal induced, 195 μg/g wet weight (15,000 *g* supernatant fraction), 19 μg/g wet weight (microsomal fraction); vitamin C deficient induced, 71 μg/g wet weight (15,000 *g* supernatant fraction), 5 μg/g wet weight (microsomal fraction).

‡ Mean ± S.E. of 10 animals per group.

§ Mean ± S.E. of 7 animals per group.

DISCUSSION

From the studies reported, it is apparent that neither over-all drug oxidation activities nor levels of microsomal electron transport components are decreased significantly in guinea pigs maintained on a vitamin C diet for 10 days, under conditions where the concentration of liver microsomal ascorbic acid was 50 per cent of normal. On the other hand, if guinea pigs were maintained on a vitamin C deficient diet for 21 days where the concentration of liver microsomal ascorbic acid reached approximately 30 per cent of normal, there was a marked decrease in over-all drug oxidation activities as well as the level and activity of individual microsomal electron transport components. The decrease observed in cytochrome P-450 and NADPH cytochrome P-450 reductase activity (40 and 85 per cent respectively) is in contrast to the studies reported by Kato *et al.*[8] These investigators found no appreciable change in electron transport components in their vitamin C deficient guinea pigs, but did find a decrease in the activity of aniline hydroxylase. It should be pointed out, however, that their animals were much larger (400 g) than those employed in this study (200–250 g) and were placed on a vitamin C deficient diet for only 12 days. Our findings are more consistent with the studies of Leber *et al.*[24] who have shown significant decreases in the demethylation of aminopyrine, the hydroxylation of acetanilide, and the quantity of cytochrome P-450 in vitamin C deficient animals. It should be pointed out that our animals on the vitamin C deficient diet for 21 days suffered no appreciable weight loss (at most 5 per cent) and were not frankly scorbutic. Furthermore, experiments with fasted animals, that had lost 25 per cent of their body weight, indicate that the effects observed in vitamin C deficiency cannot be due to decreased caloric intake, since over-all drug oxidation activities and microsomal electron transport components were either normal, or had in fact, increased 2- to 3-fold.

In contrast to the K_m studies where there was no obvious correlation between decreased drug enzyme activities and apparent affinity constants of drug substrates, consistent alteration in the usual aniline–cytochrome P-450 binding spectrum was found with vitamin C deficient guinea pig microsomes. The aniline–cytochrome P-450 binding spectrum was atypical in that the trough of the spectrum appeared at 405 nm instead of 390 nm and the peak occurred at 440 nm instead of 430 nm. There was also a marked decrease in the absorption intensity at these wavelengths. The latter finding can be explained by a concomitant decrease in the quantity of cytochrome P-450 in vitamin C deficient guinea pig microsomes. Furthermore, dilution of microsomes from normal guinea pigs to equivalent cytochrome P-450 levels found in scorbutic guinea pigs' microsomes gave the usual aniline-binding spectra with a trough at 390 nm and a peak at 430 nm. The atypical aniline–cytochrome P-450 spectrum in vitamin C deficient guinea pig microsomes may reflect an alteration in the integrity of the microsomal phospholipid membrane associated with cytochrome P-450 and ascorbic acid may be required and necessary for its maintenance. Attempts to restore over-all drug oxidation activity, NADPH cytochrome P-450 reductase activity, and altered aniline–cytochrome P-450 binding by the addition *in vitro* of ascorbic acid ($2 \cdot 3 \times 10^{-3}$ M) to vitamin C deficient liver microsomes were unsuccessful. This may not be too surprising if the integrity of the microsomal phospholipid membrane had been irreversibly damaged by the depletion of the vitamin. Attempts to replace ascorbic acid with other reducing agents such as glutathione, and 2,6-dichlorophenolindophenol dye were also ineffective. However, ascorbyl palmitate ($2 \cdot 3 \times 10^{-3}$ M), a more lipophilic analog of ascorbic acid, did restore atypical aniline–cytochrome P-450 binding spectra to the usual type in that the trough and peak of the spectrum were at 390 and 430 nm respectively; however, the absorption intensity at these wavelengths was still decreased as was over-all oxidation activities, both of which could reflect the lower quantity of cytochrome P-450 which was not restored by ascorbyl palmitate.

Reversal of decreased microsomal drug-metabolizing activities in vitamin C deficient animals by the administration *in vivo* of ascorbic acid indicate that, although the quantity of liver ascorbic acid was restored to normal levels within 3 days, over-all drug oxidation activities and levels of electron transport components were still depressed. Furthermore, the atypical aniline–cytochrome P-450 binding spectra was still present at this time. It took from 6 to 10 days of ascorbic acid administration to restore most of the drug-metabolizing activities. *O*-demethylase activity and microsomal electron transport components, such as cytochrome P-450 and NADPH cytochrome P-450 reductase, returned within 6–10 days while aniline hydroxylase activity took 3–6 days. Also, when the level of cytochrome P-450 was restored, aniline–cytochrome P-450 binding spectra also returned to a typical type II spectrum. One possibility to consider is that the time required for reversal may be due to the time needed for resynthesis of liver microsomal protein in the vitamin C deficient animal. Studies by Levin and Kuntzman[25] in the young rat have indicated that there are two rates of synthesis of the hemoprotein associated with cytochrome P-450: a fast phase which has a half-life of 7 hr, and a slow phase which has a half-life of 48 hr. Studies of rates of synthesis of this protein have not been done in young guinea pigs, and it would be of interest to determine if the time required for reversal of drug-metabolizing activities in the vitamin C deficient guinea pigs correlate with the time required for hemoprotein synthesis in this species. Our studies with phenobarbital induction indicate that the protein-synthesizing mechanism in vitamin C deficient guinea pigs is operable and not jeopardized by the depletion of vitamin C. Liver microsomal cytochrome P-450, NADPH cytochrome P-450 reductase, aminopyrine *N*-demethylase, *p*-nitroanisole, *O*-demethylase and aniline hydroxylase were all inducible in vitamin C deficient guinea pigs; in fact, the degree of induction was equal to and in some cases, as with NADPH cytochrome P-450 reductase, greater than that in normal guinea pigs.

It is interesting that under the conditions of vitamin C deficiency, all the over-all drug oxidation activities examined, as well as the level and activity of microsomal electron transport components, were reduced in the order of 50–60 per cent, except for NADPH cytochrome P-450 reductase activity which was well below 85 per cent of normal activity. The possibility should be considered that vitamin C deficient guinea pigs possess auxiliary systems which are capable of metabolizing drugs. Staudinger *et al.*[26] have recently identified a microsomal ascorbic acid dependent NADH oxidase system in rat liver, kidney and adrenals, which, as an oxido-reductive system, was capable of metabolizing such drugs as acetanilide. We have found that normal and vitamin C deficient guinea pig liver microsomes also contain NADH-ascorbic acid oxidase activity in amounts comparable to that found by Staudinger in the rat (0·24 μmoles NADH oxidized/hr/mg microsomal protein in guinea pig compared to 0·40 μmoles in the rat). This enzyme activity in vitamin C deficient guinea pig liver microsomes was found to be approximately 50 per cent of the activity of normal guinea pig

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microsomes (0·11 μmoles/hr/mg microsomal protein compared to 0·24 μmoles). This auxiliary NADH microsomal system may be an important pathway for drug metabolism in the absence of a functioning microsomal NADPH P-450 reductase system.

In an attempt to extrapolate the studies *in vitro* presented to the earlier studies *in vivo* in vitamin C deficient guinea pigs, we have found an excellent correlation of decreased microsomal drug-metabolizing activities with the findings *in vivo* of Axelrod *et al.*[5] They determined the biological half-life of aniline, antipyrine and acetanilide in the plasma of normal and vitamin C deficient animals. They also measured the time it took to reverse the increased half-life of the drug in vitamin C deficient animals by repleting the animals with ascorbic acid. The age and weight of the guinea pigs used were in the same order as ours (270 g), and they were on the vitamin C deficient diet for the same length of time (18–20 days). These investigators found that drug metabolism, as measured by the half-life of the drug in the plasma, was increased in the order of 60–70 per cent which is in reasonable agreement with our enzymatic studies *in vitro*. Of importance is the correlation in the reversal *in vivo*; for example, they found that the half-life of aniline required approximately 5–6 days to return to normal, and aminopyrine required 8 days to return to normal.

It is tempting to consider the significance of the studies presented with vitamin C deficient animals to man in that both these species cannot synthesize ascorbic acid. Although the animals were maintained on a vitamin C deficient diet for a relatively long period of time (21 days), they did not show appreciable weight loss, and frank scorbutic symptoms were not apparent. The possibility should be considered that humans, particularly in their growth period, with some deprivation of vitamin C may show sensitivity and possible toxicity to drugs. Of more importance would be the length of time required for a vitamin C deficient human to re-establish normal drug metabolism even after adequate supplements of the vitamin.

REFERENCES

1. J. R. GILLETTE, in *Forstschritte der Arzneimittelforschung* (Ed. E. TUCKER), Vol. 6, p. 11. Burkhauset Verlag, Basel (1963).
2. A. H. CONNEY, *Pharmac. Rev.* **19**, 317 (1967).
3. R. K. RICHARDS, K. KUETER and T. I. KLATT, *Proc. Soc. exp. Biol. Med.* **48**, 403 (1941).
4. R. K. RICHARDS, *Anesth. Analg.* **26**, 22 (1947).
5. J. AXELROD, S. UDENFRIEND and B. B. BRODIE, *J. Pharmac. exp. Ther.* **111**, 176 (1954).
6. A. H. CONNEY, G. A. BRAY, C. EVANS and J. J. BURNS, *Ann. N.Y. Acad. Sci.* **92**, 115 (1961).
7. E. DEGKWITZ, P. LUFT, U. PFEIFFER and H. STAUDINGER, *Hoppe-Seyler's Z. physiol. Chem.* **349**, 465 (1968).
8. R. KATO, A. TAKANAKA and T. OSHIMA, *Jap. J. Pharmac.* **19**, 25 (1969).
9. K. J. NETTER and G. SEIDEL, *J. Pharmac. exp. Ther.* **146**, 61 (1964).
10. V. G. ZANNONI, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. B. N. LA DU, H. G. MANDEL and E. L. WAY), p. 566. Williams & Wilkins, Baltimore (1971).
11. T. NASH, *Biochem. J.* **55**, 416 (1953).
12. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **94**, 22 (1948).
13. C. H. WILLIAMS and H. J. KAMIN, *J. biol. Chem.* **237**, 587 (1962).
14. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2379 (1964).
15. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2370 (1964).
16. P. L. GIGON, T. E. GRAM and J. R. GILLETTE, *Biochem. biophys. Res. Commun.* **31**, 558 (1968).
17. P. L. GIGON, T. E. GRAM and J. R. GILLETTE, *Molec. Pharmac.* **5**, 109 (1969).
18. J. R. GILLETTE, *Metabolism* **20**, 215 (1970).
19. H. REMMER, J. SCHENKMAN, R. W. ESTABROOK, H. SASAME, J. GILLETTE, S. NARASIMHULU, D. Y. COOPER and O. ROSENTHAL, *Molec. Pharmac.* **2**, 187 (1966).
20. J. H. ROE and C. A. KUETHER, *J. biol. Chem.* **147**, 399 (1943).
21. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).

22. T. E. GRAM, A. M. GUARINO, D. H. SCHROEDER, D. C. DAVIS, R. L. REAGAN and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **175**, 12 (1970).
23. Y. IMAI and R. SATO, *Biochem. biophys. Res. Commun.* **22**, 620 (1966).
24. H. LEBER, E. DEGKWITZ and H. STAUDINGER, *Hoppe-Seyler's Z. physiol. Chem.* **350**, 439 (1969).
25. W. LEVIN and R. KUNTZMAN, *J. biol. Chem.* **244**, 3671 (1969).
26. H. STAUDINGER, K. KRISCH and S. LEONHAUSER, *Ann. N.Y. Acad. Sci.* **92**, 195 (1961).

Arch. Dis. Child. 10(58): 253-264, 1935.

THE ISOLATION AND IDENTIFICATION OF VITAMIN C

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At the time when Barlow's activities were gradually impressing upon medical opinion the identity of infantile scurvy with the adult form of the disease and the fact that their common source lay in errors of diet, the science of nutrition was beginning to reveal the importance of the presence in some natural foods of small quantities of unknown substances essential to life (Lunin[40]), which were later to become known as vitamins. Neither the clinician nor the experimental investigator could then foresee the magnitude of the problem to which these observations were leading. The horizon, however, definitely widened when Grijns[19], in continuation of Eijkman's work, suggested that the cause of beri-beri was a dietetic deficiency.

That scurvy was caused by the consumption of an incomplete diet was by then almost universally acknowledged and consequently the discovery that the origin of another disease could be traced to faulty nutrition was not unique. The method of approach, on the other hand, which led to the discovery of this 'deficiency disease' was distinctly novel and was to become instrumental in the progressive activity soon to follow in this field of research.

As far back as 1869, Forster, working in Voit's laboratory at Munich, was investigating the function of mineral constituents in the diet and published his results in 1873[17]. In the course of this work he administered to pigeons a diet consisting of caseinogen and starch previously purified so as to remove the inorganic constituents with the result that the experimental birds developed symptoms, including opisthotonus, which were characteristic of what is now recognized as avian polyneuritis. The onset of this condition Forster ascribed to a deficiency of salts, not an unexpected conclusion considering the object of the inquiry and the trend of nutritional research at that period. The attention of the experimenter was then mainly focussed on the mineral constituents of the diets with the object of demonstrating what is now taken for granted, namely, their indispensability. It was, in fact, research of this character which was gradually yielding results destined to lead later to the equally gradual formulation of the vitamin hypothesis. Some twenty years after Forster's description of his experiments, similar observations, this time to be correctly interpreted, were independently made by Eijkman[12, 13, 14]. The prevalence of beri-beri amongst Europeans in the Dutch Indies impelled the Dutch Government to send out a commission in

1886 to inquire into the cause of this disease. Eijkman was one of the assistants of this commission and remained behind after its departure to pursue the inquiry. During his investigations an epidemic broke out amongst his experimental hens. The symptoms displayed were similar to those described by Forster. Eijkman, however, soon satisfied himself that these symptoms were of polyneuritic origin and he named the disease 'polyneuritis gallinarum.' Furthermore he was struck by the similarity of this disease with that of beri-beri in man which he was studying. With characteristic shrewdness, Eijkman instituted an inquiry into the origin of the epidemic. It was then revealed that during the time when the birds were suffering from polyneuritis they were fed on rejected boiled polished rice from the kitchen of the military hospital which the 'diener' of Eijkman's laboratory (under civil administration) utilized on grounds of economy. Fortunately the cook in the military hospital was replaced by one who was less favourably disposed to civilian activities and the perquisite was stopped. On the consequent re-introduction of unpolished rice the epidemic amongst the birds ceased. The cause of the epidemic was plainly suggested by this coincidence. Further work of Eijkman and his successors, and above all of Grijns[19], showed first that the deficiency of the polished-rice diet was not due to the lack of inorganic constituents and secondly that human beri-beri also resulted from the one-sided consumption of decorticated rice from which a dietetic factor had been removed in the milling. Such striking observations could not remain dormant for long.

It is outside the scope of this paper to trace the numerous and interesting investigations on experimental polyneuritis and beri-beri that followed. The lines on which these researches were pursued made circumstances propitious for the discovery of the production of experimental scurvy and as is usual in human progress they found their man. Axel Holst[31, 32] in an endeavour to find out whether polyneuritic diets were able to produce the disease in animals other than birds, observed that guinea pigs developed a pathological condition distinctly different from polyneuritis. This condition Holst and Frölich were able to identify with scurvy. Further point was lent to the problem when they found that the scorbutic symptoms failed to appear when the deficient diet was supplemented by antiscorbutic foods. Five years later they published[33] a full record of their brilliant experiments, which will always remain classical.

The results of this pioneer effort were not long in being put into practice. The provisioning of the troops in the great war, especially in certain zones, presented possibilities of 'deficiency disease' making its appearance. This necessitated the careful assessment of the vitamins in raw and treated food-stuffs. Holst and Frölich's work was extended with great rapidity and methods were improved and made quantitative in nature (Chick and Hume[6, 7]). Moreover, laboratory observations were utilized and found to be of value in the actual prevention and cure of scurvy in the field. The ground was now prepared for the task of the biochemist.

Chemical studies.

It will be easier to appreciate fully the development of the chemistry of vitamin C by recalling the appearance of the problem in the early stages of the inquiry. The only method of establishing the presence or absence of the vitamin was the biological method which, as is well known, is time consuming and often subject to untoward delays and complications. The choice of reagents that could be used in the chemical study of the vitamin was limited since toxicity had to be considered in connection with the testing. Negative results could not always be interpreted as such, because the inactivation of more or less concentrated preparations in the process of manipulation had to be considered owing to the labile nature of the vitamin. These difficulties were to a great extent overcome as the chemical properties were gradually revealed by patient research. In fact, the concentration of the active principle was only achieved in intermittent stages as its conditions of inactivation became known. Although the fractionation and the study of the chemical and physical properties occurred concurrently, for the sake of clarity the two branches of research will be treated separately and not in chronological order.

The citrus fruits were always considered as specifics against scurvy. This fact was confirmed by experiments showing the lemon and the orange to belong to the most potent natural sources known at the time. It was only comparatively recently that the mango (Perry and Zilva[42]), paprika (Svirbely and Szent- Györgyi[49]) and the anterior lobe of the pituitary (Gough and Zilva[18]) were discovered to be markedly more active. The lemon particularly appeared to lend itself to chemical work since the bulk of the solid matter in its juices, namely, the organic acids, was found to possess no antiscorbutic activity. The removal of this inactive residue yields in consequence a solution the potency of which can be raised by concentration at low temperature and pressure. Such preparations were found to be active when tested on animals and human beings (Harden and Zilva[21], Harden, Still and Zilva[20], Zilva[62, 64]). After removal of the acids from lemon juice it was shown that the sugar contained in the residue could be fermented without impairing the activity of the juice (Zilva[63], Lepkovsky, Hart, Hastings and Frazier[39]). Alcohol precipitated further impurities after which precipitation with basic lead acetate yielded a very active fraction (Zilva[65]). It is now known that these preparations sometimes approximated very closely indeed in activity to that of pure ascorbic acid, but were characterized by instability and, not being crystalline, offered little possibility of chemical identification. Lemon juice is a source from which the crystalline vitamin can be obtained only with the greatest difficulty. Later the process was modified by precipitating the active principle with neutral lead acetate at pH 7·2 (Zilva[67]). Concentrates were also obtained by basic lead acetate precipitation from other plant juices such as swede juice (Zilva[66]) and cabbage juice (Bezssonoff[4]).

Data which afforded some illuminating information on the chemical character of the antiscorbutic factor were obtained by means of diffusion experiments. Holst and Frölich had already proved that the vitamin could diffuse through a parchment membrane, which implied that the active principle was not a colloid or associated with anything in colloidal dispersion.

A technique of differential dialysis devised by Brown[5] offered further possibilities of studying this problem.

Brown's technique consisted in dialysing solutions through collodion thimbles without the application of pressure. By soaking the thimbles in alcohol of various strengths, different degrees of permeability were obtained. Thus a thimble soaked, say in 30 per cent. alcohol, allowed substances of lower molecular dimensions to pass through, whilst impeding the passage of larger molecules which could pass through a thimble soaked in stronger alcohol, such as 90 per cent. In other words a molecular sieve with meshes of varying sizes was obtained by regulating the strength of the alcohol in which the collodion was soaked. Applying this technique to the study of vitamin C (Zilva and Miura[73]) it was found that membranes which permitted a free passage to substances of small molecular dimensions such as sodium chloride retained the antiscorbutic factor during a period of three to four days. Only thimbles through which semicolloidal substances, such as dyes, passed allowed the vitamin to dialyse. In extending this investigation it was demonstrated (Connell and Zilva[9]) that the active principle diffused through membranes of somewhat lower permeability than that which permitted the passage of dyes, the size of the active molecule appeared to be not far removed from that of a hexose and further the rates of diffusion of the antiscorbutic factor, of the nitrogenous residue and of the reducing sugars were different.

These results indicated that vitamin C was in all probability nitrogen-free and that the size of the active molecule whether associated or free was that of a hexose. On the identification of the vitamin nine years later, it was found indeed to be a hexose derivative.

As mentioned above one of the greatest difficulties in the fractionation and purification of the vitamin was its lack of stability and it was therefore, of paramount importance to throw some light on the mechanism of this inactivation.

Holst and Frölich emphasized in their pioneer work the instability of vitamin C. They found, for instance, that certain vegetables (white cabbage, dandelion) lost much of their potency in the process of cooking. The juices were even more thermolabile and deteriorated quickly also at room temperature. Acid juices, on the other hand, were more stable both when heated and when stored. They demonstrated that this increase in stability was due to acidity since citric acid extracts of cabbage juice were markedly more stable than the untreated juice. Further work on the subject indicated that the loss in activity incurred during heating could not be due entirely to thermal degradation. Delf[11] observed that when swede and orange juices were heated at temperatures above 100° C. in a closed autoclave the loss of activity was much reduced. She, therefore, suggested that the rate of destruction of the vitamin was affected either directly by retarding oxidation or indirectly by the production of stabilizing bodies. That the former was the case was proved simultaneously by two workers as an indirect outcome of different investigations. Hess[26] and Hess and Unger[27] found that the addition of hydrogen peroxide to raw milk under conditions which prevented the growth of bacteria had a deleterious influence on the antiscorbutic activity of the milk, from which it was concluded that this destruction was due to oxidation especially as they also found that neutralized milk or tomato juice loses antiscorbutic value on shaking in air. The other line of evidence emerged from the study of the effect of ultraviolet light. It was found that when the ozone generated by the lamp did not come in contact with the antiscorbutic solution the activity was hardly impaired (Zilva[58]). On the other hand, contact with ozone even at room temperature or the passage of air for several hours destroyed the vitamin. When the decitrated lemon juice, used in these experiments, was boiled in an atmosphere of CO_2 for two hours no marked diminution in the antiscorbutic activity could be recorded (Zilva[59, 60]). It, therefore, became clear that oxidation was the main factor concerned in the destruction of the antiscorbutic factor. Even heating decitrated lemon juice in an autoclave for one hour at 40 lb. pressure (143° C.) destroyed only about one-half of the vitamin (Zilva[69])—a loss most probably due not to thermal degradation but to the presence of very slight traces of oxygen in solution.

The destructive effect of oxygen on vitamin C having been established it became clear that this reaction was controlled by another factor, namely, the hydrogen-ion concentration. The observations of Holst and Frölich that the antiscorbutic activity of extracts disappeared in alkaline much quicker than in acid solution could now be co-ordinated with the oxidative process of inactivation. It was shown (Zilva[61]) that decitrated lemon juice when made $\frac{N}{10}$ alkaline and exposed to the air at room temperature lost about 80 per cent. of its potency in half an hour. On the other hand, when the same solution was kept in the absence of air no loss in activity took place. Moreover such drastic treatment as aspirating air through a boiling solution of lemon juice as acid as pH 2·2 greatly delayed the destruction of the active principle. These experiments, therefore, established the fact that the destructive oxidation of the vitamin was deterred in acid and greatly accelerated in alkaline solution. Other factors conducing to the oxidation of vitamin C will receive later reference.

Another characteristic property of active solutions was their power of reducing a number of reagents. Bezssonoff[2, 3] found that vegetable extracts containing the vitamin reduced phosphomolybdotungstic acid (Folin reagent for phenols). Even concentrates from which many impurities had been removed were observed to reduce ammonical silver nitrate and to decolorize potassium permanganate in the cold (Zilva[63, 65]). These results were so striking that it became evident that they occupied a pivotal position in the scheme of vitamin C research. An early attempt to correlate these two reactions with antiscorbutic activity (Connell and Zilva[6]) failed to establish a parallelism. Both these reagents, however, are reduced by a great number of substances and consequently this negative result could not be considered as final. A more specific reagent phenolindophenol* was eventually found which made it possible to determine conveniently the reducing capacity of antiscorbutically-potent substances with precision (Zilva[68]). The reagent is decolorized by antiscorbutic solutions in the process of reduction and by this reaction the connection between reduction and vitamin activity was investigated. Two main features came to light in this inquiry. One was, that although a certain parallelism between antiscorbutic activity and reduction existed, there were, nevertheless, marked deviations. The second observation showed that when decitrated lemon juice was oxidized with indophenol and administered to the

* Substituted indophenols fulfil the same object.

experimental animals immediately, the major part of the vitamin activity was retained by the solution. If, on the other hand, the oxidized antiscorbutic was allowed to remain for about twenty-four hours before dosing it became almost inactive (Zilva[69]). The writer's interpretation of his results was that vitamin C itself did not reduce indophenol but that the decolorization of the indicator was due to a reducing substance closely associated with the active principle, which tended to prevent oxidation.

About this time the hope of isolating the vitamin was realized but in a manner which had not been foreseen. Moreover, the antiscorbutic character of the substance thus isolated was not appreciated until some years later. Engaged in an investigation on the part played by the adrenal cortex in biological oxidation with the ultimate aim of elucidating its function in the renal system, Szent-Györgyi[50] isolated from the cortex a highly reducing hexose derivative—hexuronic acid. This compound he found also in vegetables and fruits where it appeared to function in connection with peroxidase systems, thus associating the adrenal cortex with this oxidizing mechanism. Amongst other observations he recorded that hexuronic acid decolorized indophenol—a fact which prompted him to suggest that the acid was probably identical with the reducing substance postulated by the writer (Zilva[68]). Professor Szent-Györgyi kindly offered to supply the writer with hexuronic acid in order to test this suggestion experimentally, but unfortunately owing to the scarcity of the material at the time and his subsequent departure from England the matter was left in abeyance. However, another substance isolated from orange juice by Szent-Györgyi, which he thought might be the vitamin (private communication by letter) was in the meantime (June-July, 1928) tested by the writer at Professor Szent-Györgyi's request and was found to be antiscorbutically inactive in daily doses of 3 mgm. and 5 mgm. (cf. Svirbely and Szent-Györgyi[46]).

Not many years elapsed before the chemical identity of vitamin C was established and, as in the case of its isolation, in a manner not expected. Tillmans*, in trying to devise a method for discriminating between natural lemon juice and the artificial product employed 2, 6-dichloroindophenol, it being known that this compound was decolorized by orange juice, with the result that he found that only the fresh natural lemon juice reduced the indicator. In communicating this observation at a meeting in Nüremberg the point was raised whether this reducing capacity had any connection with vitamin C. On investigating this matter further he found[51], as the writer had done before, a certain parallelism between the two properties, but unlike the writer, he was inclined to attribute the two properties to one substance. The writer's observation that decitrated lemon juice retained its antiscorbutic activity after oxidation, Tillmans maintained could be explained by assuming that the substance was reversibly oxidized whilst remaining antiscorbutically

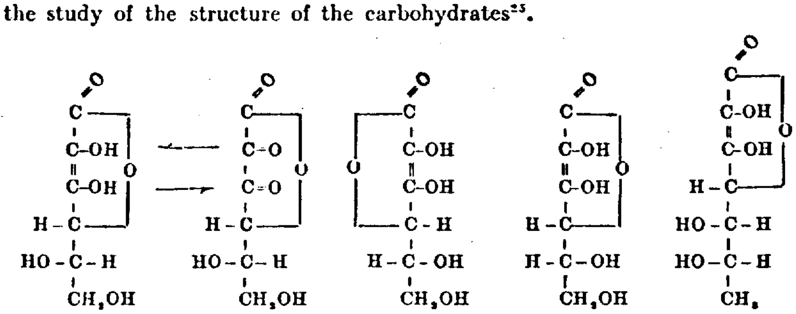
* Whilst writing this review (February, 1935) news reached me of Professor Tillmans' death. I should like to take this opportunity of expressing my sincere admiration not only of his capacity bu' also of his truly scientific spirit.

active. In this first stage of oxidation, according to him, the vitamin was more prone than in the original reduced form to destruction by further oxidation. It is of interest to note that Szent-Györgyi[50] had found that hexuronic acid could also be reversibly oxidized but his work was not known to Tillmans in 1930. The similarity, however, between the reducing substance which Tillmans considered to be vitamin C and hexuronic acid was thus indicated then. Later (Tillmans et al.,[52, 53, 54, 55, 56]) when he had worked out his thesis in greater detail and became acquainted with the work on hexuronic acid he actually suggested that vitamin C was probably identical with it. Very soon after the appearance of Tillmans' last series of publications, King and Waugh[38, 37] claimed to have isolated a crystalline substance from lemon juice, by the decitration and lead acetate method mentioned previously, which, in their opinion, was hexuronic acid and which was antiscorbutically active. Simultaneously Szent-Györgyi[47, 48] examined an authentic sample of crystalline hexuronic acid from the adrenal cortex and found it also potent.

The antiscorbutic activity of hexuronic acid could not, however, be taken as a final proof that this compound was identical with vitamin C (Zilva[72]). Workers in the vitamin field were particularly reminiscent of precedents in which active crystalline substances owed the particular vitamin potency not to the crystalline compound but to adhering 'impurities.' A similar condition could be visualized in the case of hexuronic acid. Crucial evidence was nevertheless soon obtained which militated against this view. Szent-Györgyi[49] was able to show that the monoacetone derivative of ascorbic acid—under which name hexuronic acid became at this stage to be known—was antiscorbuticallly active but not to the full extent and that the ascorbic acid recovered from it was, on the other hand, fully active. Hirst and Zilva[28] found that when ascorbic acid was oxidized with iodine to its first oxidation product (dehydroascorbic acid) it was almost as active as it was before oxidation. This observation was similar to the one made by the writer[68] when the vitamin in decitrated lemon juice was oxidized with indophenol. By reducing the dehydroascorbic acid, ascorbic acid was re-obtained which was as active antiscorbutically as the original compound from which the oxidation product was prepared. These results strongly pointed to the fact that ascorbic acid was active per se. The alternative explanation that the vitamin was in association with ascorbic acid and could, like it, be reversibly oxidized and regenerated quantitatively was in the highest degree improbable. In view of these facts the writer's hypothesis that the reducing substance and the vitamin were not identical but in close association became untenable and Tillmans' theory was thus shown to be correct. The observation that synthetic ascorbic acid obtained from inactive material was fully active (Reichstein and Oppenhauer[45], Haworth, Hirst and Zilva[24]) supplied the final and incontrovertible proof.

Structure of the vitamin.—As was to be expected a compound of such physiological significance as ascorbic acid could not fail to engage the attention of carbohydrate chemists. An intensive quest led to the establishment of its chemical structure and the eventual realization of its synthesis. Only the outstanding achievements can be given here in the barest outline.

Of the several structural formulae proposed the one which has found general acceptance is that shown below. This was put forward by Hirst and his collaborators of Birmingham, whose proof of this structural formula was arrived at by the ingenious application of various methods employed in the study of the structure of the carbohydrates[25].



l-ascorbic acid	dehydro-ascorbic acid	*d*-ascorbic acid	*d*-arabo-ascorbic acid	*l*-rhamno-ascorbic-acid
active	active	not active	active $\frac{1}{20}$	active $\frac{1}{5}$

A little later the same formula was suggested independently by Euler and Martius[15], their suggestions being based on indirect analogy with the substance reductone (CHOH:COH·CHO), which resembles ascorbic acid in its property of reducing indophenol. The synthesis of ascorbic acid was soon accomplished independently by Reichstein of Zurich[45] and by Haworth, Hirst and their school at Birmingham[24] from *l*-xylosone by way of the hydrogen cyanide addition compound. As xylosone is difficult to prepare this method of synthesis could not conveniently be applied in the preparation of large quantities of ascorbic acid. New methods have been developed in which the starting material is *l*-sorbose, a sugar which is now readily available in quantity. In Reichstein's method[44] sorbose is converted into its acetone compound and thence into 2-keto-*l*-gulonic acid, from which *l*-ascorbic acid is easily obtained (compare Maurer and Schiedt's transformation of methyl 2-keto-*d*-gluconate into *d*-arabo-ascorbic acid[41]). In Haworth's method[22, 23] the requisite 2-keto-*l*-gulonic acid is produced directly by simple oxidation of *l*-sorbose. By such methods *l*-ascorbic acid can now be produced at an economic price.

The schemes employed in the synthesis of *l*-ascorbic acid were eventually utilized in the preparation of a number of analogues and derivatives, of which some such as *d*-arabo-ascorbic acid (Maurer and Schiedt[41], Dalmer and Moll[10]), *l*-rhamno-ascorbic acid (Reichstein[43], Reichstein, Schwarz and Grüssner[46]) and *l*-gluco-ascorbic acid (Reichstein[43]) have been found to be partly potent, whilst others had no antiscorbutic activity. As a striking example of these latter isomers one may mention *d*-ascorbic acid, the enantiomorph of the fully active *l*-ascorbic acid.

Chemical and physical properties.

The availability of the pure compound made it possible to study its chemical and physical properties in greater detail. The characteristics of the vitamin previously established were fully confirmed. One feature, however, calls for comment. Pure crystalline *l*-ascorbic acid and the other active compounds are perfectly stable in solid condition, a fact which seems at first to be strikingly contradictory to the instability of vitamin C. This apparent paradox can nevertheless be explained. In studying the process of inactivation of the antiscorbutic factor it was found (Zilva[69, 70, 71], Johnson and Zilva[34]) that certain phenolic substances, which are widespread in plant tissues, are subject to spontaneous oxidation giving rise to products capable of oxidizing in their turn the vitamin. In addition, Euler, Myrbäck and Larsson[16] showed that traces of certain metals could catalyse the oxidation of ascorbic acid, an observation which explains why the pure compound becomes slowly inactivated in aqueous solution. Recent work throws further light on this subject. In water carefully distilled from and received in quartz apparatus, *l*-ascorbic acid is exceptionally stable even when this water is previously saturated with oxygen (Kellie and Zilva[36]). This suggests that contact with oxygen in the absence of catalysts does not destroy the activity. The instability of the vitamin in natural juices or in concentrates when exposed to air is evidently due to the presence of various catalytic substances. That mere traces of these impurities may have a deleterious effect on the vitamin is seen from the fact mentioned above that concentrates were sometimes obtained by the writer which, weight for weight, were almost, if not as active, as crystalline ascorbic acid, but lacking the stability of the latter. The natural medium from which these preparations were made, namely, lemon juice, offered great technical difficulties in removing the adhering traces of these destructive impurities which imparted the instability to the vitamin in this condition.

It is significant that in the tissue of the living plant or animal biological conditions are such as to conduce to the stability of vitamin C. Only when the tissue is disintegrated is this balance upset. Since in the natural sequence of things the disintegration takes place through mastication as the food is consumed, no serious loss in antiscorbutic activity can take place before the ingested vitamin is utilized by the organism.

As in natural sources and in concentrates indophenol still remains the most specific reagent for pure *l*-ascorbic acid and its analogues. Nevertheless the specificity is far from being complete, so that this reagent is of doubtful value for detection and determination of these compounds. Since his introduction of this indicator in 1927 the writer had the opportunity of carrying out a critical study of this problem on a large scale, especially

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between 1928 and 1933, during which time he and his colleagues were engaged in an extensive inquiry into the vitamin C of natural products. He arrived at the conviction that in spite of the excellent agreement between the biological and the indophenol titration values found in many instances, the latter procedure broke down in unexpected instances in a way which made the general application of the indicator in the assessment of antiscorbutic potency undesirable, particularly in the hands of the less cautious. For this reason he deliberately abstained from urging the utilization of this reaction only for the detection of the antiscorbutic factor. The same criticism may be levelled at the application of the selective absorption displayed by ascorbic acid in the ultraviolet region of the spectrum for the determination of vitamin C. The biological activity still remains the ultimate criterion for vitamin C. Among the biological methods the prophylactic method yields the most accurate results but is laborious. The curative method in which the test dose is administered to the animals from the tenth to fifteenth day on the basal diet until the thirtieth day when they are killed is almost as accurate and less laborious (cf. Johnson and Zilva[35]). Another biological method—Höjer's method—is sometimes employed. This method is based on the fact that in guinea-pigs on a scorbutic diet there is an early change in the odontoblastic layer of the teeth (Zilva and Wells[74], Höjer[29,30], Key and Elphick[37]). The advantages claimed for this method, namely the small amount of material required, and the short duration of the test are possessed by the curative method which does not, on the other hand, share the disadvantages of the former. The chief of these are variability in the individual response of the experimental animals, the totally subjective interpretation of the results and the necessity of making histological sections.

In this review the chemical nature of vitamin C only has been briefly discussed. That this vital principle, the absence of which is responsible for the production of scurvy in animals incapable of synthesizing it, may play an important part in the metabolic functions of the animal organism needs little stressing. Nor is it likely that its synthesis and presence in the plant kingdom is without significance. A good deal of sound information bearing on these points which falls outside the aim of the present review has been already obtained in the course of study of the chemical nature of the antiscorbutic factor. The identification of the vitamin naturally quickened the pace of research in this domain, but it is not easy at the moment to assess the true value of many of these latter contributions in an excessively prolific field, especially as some results leave a debatable zone of possible error. Many gaps remain to be filled before a major hypothesis can be formulated. When the time, whether it be close or remote, is ripe for the integration of the essential and established facts in their true perspective, scientific workers might with advantage look back to Barlow's Bradshaw Lecture as a model for clarity of vision, logical exposition and, above all, intellectual probity.

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REFERENCES.

1. Ault, R. G., *et. al.*, *J. Chem. Soc.*, Lond., 1933, 1419.
2. Bezssonoff, N., *Compt. rend. Acad. de sci.*, Paris, 1921, CLXXIII, 466.
3. *Idem*, *Bull. Soc. chim. biol.*, Paris, 1922, IV, 83.
4. *Idem*, *Compt. rend. Acad. de sci.*, Paris, 1925, CLXXX, 970.
5. Brown, W., *Biochem. J.*, Lond., 1915, IX, 591.
6. Chick, H., & Hume, E. M., *Tr. Roy. Soc. Trop. Med. & Hyg.*, Lond., 1916-17, X, 141.
7. *Idem*, *Proc. Roy. Soc. Lond.*, Lond., 1917-9, Series B, XC, 44.
8. Connell, S. J. B., & Zilva, S. S., *Biochem. J.*, Lond., 1924, XVIII, 638.
9. *Idem*, *loc cit.*, 641.
10. Dalmer, O., & Moll, T., *Ztschr. f. physiol. Chem.*, Berlin, 1933, CCXXII, 116.
11. Delf, M., *Biochem. J.*, Lond., 1920, XIV, 211.
12. Eijkman, C., *Virchows Arch., f. path. Anat.*, Berlin, 1897, CXLVIII, 523.
13. *Idem*, *ibid.*, CXLIX, 187.
14. *Idem*, *Arch. f. Schiffs u. Tropen Hyg.*, Leipzig, 1897, I, 268.
15. Euler, H., & Martius, C., *Ark. Kemi Min. Geol.*, Uppsala, 1933, XI B, 14.
16. Euler, H., Myrbäck, K., & Larsson, H., *Ztschr. f. physiol. Chem.*, Berlin, 1933, CCXVII, 1.
17. Forster, J., *Ztschr. f. Biol.*, Munich, 1873, IX, 297.
18. Gough, J., & Zilva, S. S., *Biochem. J.*, Lond., 1933, XXVII, 1279.
19. Grijns, G., *Geneesk. tijdschr. v. Nederl. Indie*, Java, 1901, XLI, 3.
20. Harden, A., Still, G. F., & Zilva, S. S., *Lancet*, Lond., 1919, i, 17.
21. Harden, A., & Zilva, S. S., *Biochem. J.*, Lond., 1918, XII, 259.
22. Haworth, W. N., *Nature*, Lond., 1934, CXXXIV, 724.
23. Idem, *Rep. Brit. Ass. Advancement Sci.*, 1934, 295.
24. Haworth, W. N., Hirst, E. L., & Zilva, S. S., *J. Chem. Soc.*, Lond., 1934, 1155.
25. Herbert, R. W., *et al.*, *ibid.*, 1933, 1270.
26. Hess, A. F., *Brit. Med. J.*, Lond., 1920, ii, 154.
27. Hess, A. F., & Unger, L. J., *Proc. Soc. Exper. Biol. & Med.*, Utica, 1921, XVIII, 143.
28. Hirst, E. L., & Zilva, S. S., *Biochem. J.*, Lond., 1933, XXVII, 1271.
29. Höjer, J. A., *Acta Paediat.*, Uppsala, 1924, Supp. III.
30. *Idem*, *Brit. J. Exp. Path.*, Lond., 1926, VII, 356.
31. Holst, A., *J. Hyg.*, Lond., 1907, VII, 619.
32. Holst, A., & Frölich, T., *loc. cit.*, 634.
33. *Idem*, *Ztschr. f. Hyg. u. Infektionskr.*, Berlin, 1912, LXXII, 1.
34. Johnson, S. W., & Zilva, S. S., *Biochem. J.*, Lond., 1932, XXVI, 871.
35. *Idem*, *ibid.*, 1934, XXVIII, 1393.
36. Kellie, A. E., & Zilva, S. S., *ibid.*, 1935, XXIX, 1028.
37. Key, K. M., & Elphick, G. K., *Biochem. J.*, Lond., 1931, XXV, 888.
38. King, C. G., & Waugh, W. A., *Science*, New York, 1932, LXXV, 357.
39. Lepkovsky, S., *et al.*, *J. Biol. Chem.*, Balt., 1925, LXVI, 49.
40. Lunin, N., *Ztschr. f. physiol. Chem.*, Berlin, 1881, V, 31.
41. Maurer, K., & Schiedt, B., *Ber. d. deut. Chem. Gesell*, Berlin 1933, LXVI, 1054.
42. Perry, E. O. V., & Zilva, S. S., *E.M.B. Prelim. Rep. on the Vitamin Content of the Mango*, 1932.
43. Reichstein, T., *Nature*, Lond., 1931, CXXXIV, 724.
44. Reichstein, T., & Grüssner, A., *Helv. Chim. Acta*, Basel, 1934, XVII, 311.
45. Reichstein, T., Grüssner, A., & Oppenhauer, R., *ibid.*, 1933, XVI, 1019.
46. Reichstein, T., Schwartz, L., & Grüssner, A., *ibid.*, 1935, XVIII, 353.
47. Svirbely, J. L., & Szent-Györgyi, A., *Nature*, Lond., 1932, CXXIX, 576, 690.
48. *Idem*, *Biochem. J.*, Lond., 1932, XXVI, 865.
49. *Idem*, *ibid.*, 1933, XXVII, 279.
50. Szent-Györgyi, A., *ibid.*, 1928, XXII, 1387.
51. Tillmans, J., *Zeit. f. Untersuch. der Lebensmitt.*, Berlin, 1930, LX, 84.

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52. Tillmans, J., Hirsch, P., & Dick, H., *ibid.*, 1932, LXIII, 287.
53. Tillmans, J., Hirsch, P., & Hirsch, W., *loc. cit.*, 1.
54. Tillmans, J., Hirsch, P., & Jackisch, J., *loc. cit.*, 241.
Idem, loc. cit., 276.
55. Tillmans, J., Hirsch, P., & Siebert, F., *loc. cit.*, 21.
56. Tillmans, J., Hirsch, P., and Vaubel, R., *ibid.*, 1933, LXV, 145.
57. Waugh, W. A., & King, C. G., *J. Biol. Chem.*, Balt., 1932, XCVII, 325.
58. Zilva, S. S., *Biochem. J.*, Lond., 1919, XIII, 164.
59. *Idem, Lancet*, Lond., 1921, i, 478.
60. *Idem, Biochem. J.*, Lond., 1922, XVI, 42.
61. *Idem, ibid.*, 1923, XVII, 410.
62. *Idem, loc. cit.*, 416.
63. *Idem, ibid.*, 1924, XVIII, 182.
64. *Idem, loc. cit.*, 186.
65. *Idem., loc. cit.*, 632.
66. *Idem, ibid.*, 1925, XIX, 589.
67. *Idem, ibid.*, 1927, XXI, 354.
68. *Idem, loc. cit.*, 689.
69. *Idem, ibid.*, 1928, XXII, 779.
70. *Idem, ibid.*, 1929, XXIII, 1199.
71. *Idem, ibid.*, 1930, XXIV, 1687.
72. *Idem, Nature*, Lond., 1932, CXXIX, 690, 943.
73. Zilva, S. S., & Miura, M., *Biochem. J.*, Lond., 1921, XV, 422.
74. Zilva, S. S., & Wells, F. M., *Proc. Roy. Soc. B.*, Lond., 1919, XC, 505.

J. Allergy Clin. Immunol. 51:218-226 1973

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Inhibition of histamine-induced airway constriction by ascorbic acid

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We studied the effect of ascorbic acid on histamine-induced airway constriction in 17 healthy subjects; we also investigated its effect on guinea pig tracheal strips in vitro. *Ventilatory function was measured by recording partial expiratory flow-volume (PEFV) curves on which maximum flow rates at 50 per cent VC and at 25 per cent VC were calculated. Following oral administration of 500 mg. ascorbic acid, the mean reductions of $\dot{V}_{max}$ at 50 per cent VC and $\dot{V}_{max}$ at 25 per cent VC after histamine inhalation were significantly smaller in comparison with placebo administration ($P < 0.01$). In the guinea pig trachea preparation, ascorbic acid reduced contractions induced by histamine and relaxed this tissue in the absence of other agents. Propranolol did not block the effect of ascorbic acid in man (80 mg. orally), but* in vitro *relaxations of tracheal strips by ascorbic acid were reduced by 2.5 μg propranolol. Ascorbic acid probably has a direct effect on airway smooth muscle; in the guinea pig trachea its effect may be mediated by β-adrenergic receptors.*

In 1804, Reisseissen[1] described airway smooth muscle in the human lung and attributed "convulsive asthma" to its contraction. The same author[2] later observed symptoms of "convulsive asthma" in patients with severe scurvy. More recently, Dawson and West[3] reported that ascorbic acid protects guinea pigs against anaphylactic shock. These observations suggest that ascorbic acid might have a therapeutic effect in asthma, but clinical reports on this subject are contradictory.[4,5]

We have found that ascorbic acid can inhibit the airway constrictor effect of histamine in healthy human subjects and also in the isolated trachea of the guinea pig. Thus, the therapeutic use of ascorbic acid in asthma may warrant further investigation.

SUBJECTS AND METHODS

Experiments in man

Seventeen healthy subjects (14 men, 3 women, 21 to 38 years of age; 12 nonsmokers and 5 regular smokers) participated in the study. Each subject inhaled identical histamine aerosols on 2 days, a "placebo day" and an "ascorbic acid day." The aerosols were produced by a Dautrebande[6] D-30 nebulizer with an air pressure of 15 p.s.i. Histamine concentrations in

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Supported, in part, by USPHS Grants HL-14179 and HL-14534 from the National Heart and Lung Institute, and OH-00304 from the National Institute for Occupational Safety and Health.

Received for publication Aug. 22, 1972.

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the aerosolized liquid varied from 15 to 40 mg. per milliliter for different subjects (histamine dihydrochloride dissolved in 0.9 per cent saline; pH = 7.4; doses expressed as mg. base per milliliter). These were chosen on the basis of symptoms and reduction of ventilatory function during a preceding series of experiments with different histamine concentrations. Identical conditions of inhalation were used in all experiments. The duration of inhalation was 30 seconds, and on each day inhalations were performed at 9 A.M., 12 noon, and 3 P.M. (hereafter referred to as 0, 3, and 6 hours, respectively). Ventilatory function was measured immediately after inhalation (0′), and again after 2′, 4′, and 6′, by recording PEFV curves.[6] The subject inspired to about 70 per cent vital capacity and then performed a forced expiratory maneuver to residual volume (RV), immediately followed by a maximal inspiration to total lung capacity (TLC). Expiratory flow-volume curves were recorded on a Brush 500 High Performance XY Recorder (Gould, Inc.), with lung volume changes on the abscissa and expiratory flow rate on the ordinate. Maximum flow rates at 50 per cent and 25 per cent of the control vital capacity (i.e., VC at 0 hour) were measured from the PEFV curves. On each curve a constant volume (50 per cent and 75 per cent of the control VC) was subtracted from total lung capacity (TLC) and expiratory flow was read at these volumes (Fig. 1). Three control blows were made before histamine inhalation, and the mean of the 2 curves with the highest flows was used.

The comparison of maximum flow assumes that TLC is not changed by the inhalation of histamine. We confirmed that this is so by determining TLC separately in an air-conditioned volume-displacement body plethysmograph before and after histamine inhalation (30 mg. and 20 mg. per milliliter). No changes were recorded in TLC, while residual volume (RV) was increased (2 subjects).

After the first histamine inhalation (0 hour) on the ascorbic acid day, 500 mg. of ascorbic acid dissolved in water was administered orally. Histamine inhalation and ventilatory function measurements were repeated 3 and 6 hours later. After 4 to 5 days, the same procedure was repeated with a placebo identical in taste to ascorbic acid solution (water with acetic acid added). At the time of the ventilatory function measurements, heart rate was also measured.

The t test for paired samples was used for statistical analysis.

Experiments in guinea pigs

Sixteen female guinea pigs (Hartley strain; 200 to 250 grams) were used for the spirally cut trachea preparations.[8] The strips of tissue were suspended in 10 ml. Tyrode's solution at 37° C. and constantly aerated with 95 per cent O_2 and 5 per cent CO_2. Contractions and relaxations were recorded isometrically on a pen recorder. Drugs were dissolved in Tyrode's solution, and ascorbic acid was neutralized to pH 6.5 to 7.5 with sodium bicarbonate. The buffering capacity of Tyrode's solution is sufficient to prevent pH changes on addition of the ascorbic acid solution. Final bath pH was 7.4 in the experiments reported here. In separate experiments, similar results were obtained with pH values ranging from 7.2 to 7.6, kept constant throughout each experiment. Doses of drugs are expressed in μg base per milliliter final bath volume.

RESULTS

Experiments in man

Histamine aerosol inhalation causes a decrease of expiratory flow rates on the PEFV curve (Fig. 1, *A*). After pretreatment with ascorbic acid, flow rates decreased less after inhalation of the same histamine aerosol dose (Fig. 1, *B*).

The measurement of maximum expiratory flow rates at constant lung volumes ($\dot{V}_{max}$ 50 and $\dot{V}_{max}$ 25) allows a quantitative assessment of these drug-induced changes (Table I). Under control conditions (0 hour) the reduction of $\dot{V}_{max}$ 50 and $\dot{V}_{max}$ 25, after histamine inhalation, was similar on both days (with placebo or ascorbic acid). Following the administration of ascorbic acid, histamine

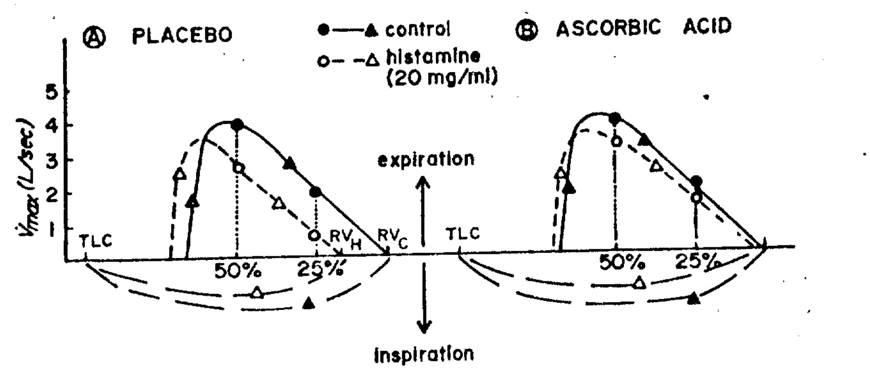

FIG. 1. PEFV curves from healthy subject on placebo day (**A**) and on ascorbic acid day (**B**). Lung volume (abscissa) in percentage of vital capacity (**TLC**, 100 per cent; **RV**, 0 per cent). **RV$_c$** = residual volume on control curves; **RV$_H$** = residual volume after histamine. Ordinate: Expiratory flow rate (L./sec). Measurement of $\dot{V}_{max}$ at 50 per cent VC and $\dot{V}_{max}$ at 25 per cent VC indicated by ● for control and O after histamine inhalation.

TABLE I. Maximum expiratory flow rates on PEFV curves

	Time (hr.)	V_{max} at 50% VC (L./sec.) Control ± S.E.*	After histamine (min.) 0′	2′	4′	6′	$\dot{V}_{max}$ at 25% VC (L./sec.) Control ± S.E.*	After histamine (min.) 0′	2′	4′	6′
Placebo day	0	4.20 ± 0.34	2.61	2.64	2.86	3.06	1.78 ± 0.23	1.05	0.97	1.15	1.26
	3	3.94 ± 0.29	2.56	2.84	2.71	2.97	1.67 ± 0.20	1.01	1.07	1.12	1.04
	6	3.85 ± 0.27	2.54	2.55	2.73	3.02	1.57 ± 0.18	0.88	0.93	1.11	1.08
Ascorbic acid day	0	4.49† ± 0.28	2.71†	2.96†	3.19†	3.19†	1.87† ± 0.18	0.99†	1.12†	1.22†	1.12†
	3	4.06† ± 0.20	2.99†	3.49‡	3.58‡	3.77‡	1.61† ± 0.15	1.13†	1.36‡	1.51‡	1.58‡
	6	3.77† ± 0.19	3.12‡	3.28‡	3.47‡	3.53‡	1.51† ± 0.13	1.19‡	1.26‡	1.32‡	1.45‡

*Mean ± standard error.
†Not significantly different from the corresponding value on the placebo day ($P > 0.05$).
‡Significantly different ($P < 0.01$) from corresponding value on placebo day.

reduced flow rates less than after administration of placebo, and the differences were statistically significant in all but one instance (the 0′ value at 3 hours).

Figs. 2 and 3 represent the data in all subjects, expressed as a percentage of the control values at 0, 3, and 6 hours, on both days. After placebo, the effect of histamine on $\dot{V}_{max}$ 50 and $\dot{V}_{max}$ 25 remains unchanged at 3 and 6 hours. After ascorbic acid the effect of histamine is reduced, and the reduction is maintained 6 hours after ascorbic acid ingestion. Although ascorbic acid reduced the effect of histamine, the decreases of flow rates were in most instances still significant compared to the control value before histamine inhalation.

On the placebo day, as well as on the ascorbic acid day, control values for $\dot{V}_{max}$ 50 and $\dot{V}_{max}$ 25 were lower at 3 and 6 hours than at 0 hours (Table I).

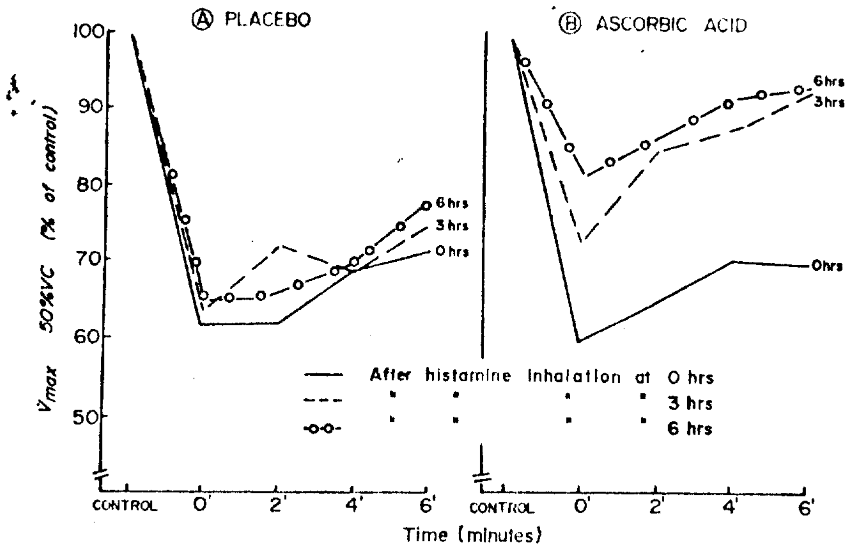

FIG. 2. Mean changes (all subjects) in $\dot{V}_{max}$ at 50 per cent VC on PEFV curves after histamine inhalation as a percentage of control values, on placebo day (**A**) and on ascorbic acid day (**B**).

TABLE II. Control flow rates in 10 subjects

Time	$\dot{V}_{max}$ at 50% VC (L./sec.)			$\dot{V}_{max}$ at 25% VC (L./sec.)		
	Placebo day ($\bar{X} \pm$ S.E.)	Ascorbic acid day ($\bar{X} \pm$ S.E.)	Day without drugs ($\bar{X} \pm$ S.E.)	Placebo day ($\bar{X} \pm$ S.E.)	Ascorbic acid day ($\bar{X} \pm$ S.E.)	Day without drugs ($\bar{X} \pm$ S.E.)
9 A.M.	3.64 ± 0.37	3.98 ± 0.35	3.39 ± 0.23	1.44 ± 0.20	1.54 ± 0.19	1.21 ± 0.21
12 noon	3.64 ± 0.42	3.74 ± 0.21	3.23 ± 0.26	1.45 ± 0.23	1.40 ± 0.17	1.10 ± 0.21
3 P.M.	3.48 ± 0.35	3.56 ± 0.23	3.49 ± 0.26	1.34 ± 0.27	1.43 ± 0.14	1.26 ± 0.22

$\bar{X} \pm$ S.E. = Mean ± standard error.

Since this might be a result of a circadian rhythm in ventilatory function, we restudied 10 of our subjects, without administration of drugs, at the same 3 hour intervals. Table II compares the average control flow rates on the 3 days of study in these 10 subjects. Only minor and insignificant changes of flows occurred on the day without any drugs.

Heart rate increased consistently after histamine inhalation, and this was not changed by administration of ascorbic acid. The increase was most marked (117 to 140 per cent of control value) immediately after histamine inhalation. Six minutes after inhalation the heart rate had returned to control values.

We tested the effect of propranolol on the protective action of ascorbic acid in 4 subjects. After 3 control blows, histamine aerosol was inhaled (15 to 20 mg. per milliliter), and PEFV curves were recorded immediately after inhalation and 2, 4, and 6 minutes later. Propranolol was then given, 80 mg. orally.

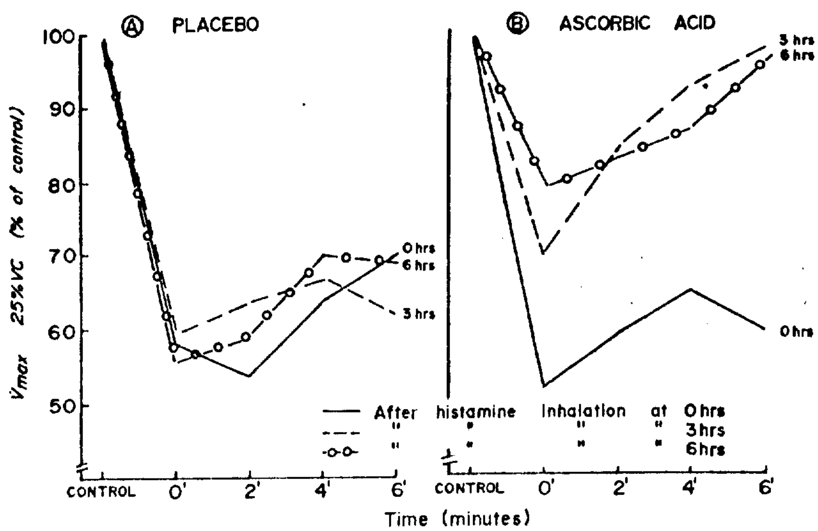

FIG. 3. Mean changes (all subjects) in $\dot{V}_{max}$ at 25 per cent VC on PEFV curves after histamine inhalation as a percentage of control values, on placebo day (A) and on ascorbic acid day (B).

After 75 minutes, the histamine inhalation was repeated and flow-volume curves recorded according to the same schedule. Ascorbic acid was then administered in a dose of 500 mg., and histamine inhalation was repeated 2 and 3 hours after ascorbic acid.

After propanolol, the effect of histamine was enhanced (Table III). This potentiation of the histamine effect was abolished by administration of ascorbic acid, which resulted in a histamine response similar to the one before administration of propranolol. Since oral propranolol in this dose acts during several hours,[9, 10] and the heart rate remained below control values throughout the experiments, we conclude that ascorbic acid can exert its protective action in the presence of β-receptor blockade by propranolol.

Guinea pig experiments

Ascorbic acid produced dose-related relaxations of the uncontracted preparation (Fig. 4, *A*), with a threshold concentration of 35 to 55 μg per milliliter. Tachyphylaxis was observed when concentrations higher than 100 μg per milliliter were used. With 1 to 25 μg per milliliter doses of ascorbic acid, no effect on the baseline of the uncontracted trachea was observed. These low doses also did not affect histamine contractions produced immediately after treatment. Ascorbic acid did produce dose-related relaxations in preparations previously contracted by submaximal concentrations of histamine (1 μg per milliliter; Fig. 4, *A*). The threshold concentration for this effect varied from 20 to 30 μg per milliliter.

When ascorbic acid was added to the organ bath, the muscle first relaxed. After the ascorbic acid was washed out, the muscle returned to the original

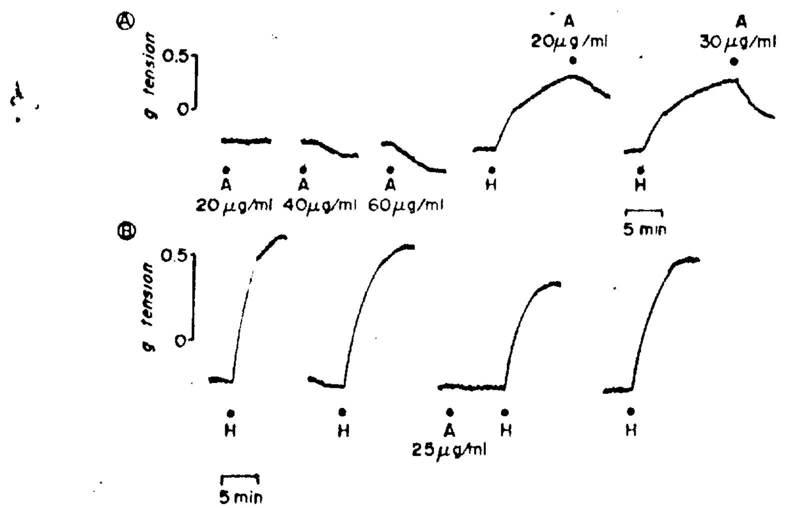

FIG. 4. Top, Effect of ascorbic acid (A) on uncontracted and contracted trachea of the guinea-pig, in vitro. Contractions are produced by 1 μg per milliliter histamine (H). **Bottom,** Effect of subthreshold ascorbic acid (A) on histamine (1 μg per milliliter, H) contractions.

TABLE III. Effect of propranolol and ascorbic acid on airway smooth muscle contraction after histamine inhalation

	$\dot{V}_{max}$ at 50% VC (L./sec.)					$\dot{V}_{max}$ at 25% VC (L./sec.)				
	Control ± S.E.*	After histamine (min.) 0′	2′	4′	6′	Control ± S.E.*	After histamine (min.) 0′	2′	4′	6′
Histamine	4.21 ± 0.60	3.45 ± 0.62	3.53 ± 0.50	3.62 ± 0.46	3.86 ± 0.48	1.85 ± 0.47	1.48 ± 0.41	1.61 ± 0.47	1.35 ± 0.41	1.68 ± 0.46
% of control		81.9	83.8	86.0	91.7		80.0	87.0	73.0	90.8
Heart rate	82	92	88	86	83					
Propranolol	4.16 ± 0.68	2.73 ± 0.86	3.30 ± 0.75	3.05 ± 0.53	3.58 ± 0.67	1.78 ± 0.39	1.15 ± 0.39	1.68 ± 0.52	1.33 ± 0.52	1.63 ± 0.51
% of control		65.6	79.3	73.3	86.0		64.6	94.4	74.7	91.6
Heart rate	70	73	71	66	67					
Ascorbic acid†	4.15 ± 0.68	3.63 ± 0.53	3.77 ± 0.53	3.72 ± 0.69	3.93 ± 0.69	1.85 ± 0.46	1.50 ± 0.35	1.68 ± 0.54	1.75 ± 0.36	1.65 ± 0.52
% of control		87.5	90.8	89.6	94.7		81.1	90.8	94.6	89.2
Heart rate	68	74	69	70	68					
Ascorbic acid‡	4.08 ± 0.62	3.82 ± 0.60	4.12 ± 0.74	3.85 ± 0.54	4.08 ± 0.65	1.77 ± 0.50	1.70 ± 0.45	1.90 ± 0.48	1.86 ± 0.50	1.92 ± 0.37
% of control		93.6	101.0	94.4	100.0		96.0	107.3	105.1	108.5
Heart rate	78	75	71	70	72					

*Mean ± standard error.
†Mean changes 2 hours after ascorbic acid.
‡Mean changes 3 hours after ascorbic acid.

baseline in 20 to 25 minutes. When ascorbic acid was added to the previously contracted preparation, the muscle returned to the original baseline somewhat more quickly (5 to 10 minutes).

Prior administration of ascorbic acid in concentrations of 30 to 50 μg per milliliter reduced contractions mediated by histamine by 20 to 45 per cent (Fig. 4, *B*).

Propranolol (2.5 μg per milliliter incubated for 10 minutes) completely prevented relaxation mediated by isoproterenol on strips previously contracted by histamine. The same dose of propranolol reduced the relaxation produced by ascorbic acid both in the histamine-contracted and in the uncontracted trachea (6 preparations). Propranolol was effective for at least 4 hours.

DISCUSSION

The measurement of maximum expiratory flow rates on PEFV curves allows a sensitive assessment of relatively slight degrees of airway constriction induced by pharmacologic agents.[7] Changes of these flow rates reflect caliber changes in small airways, for instance, in bronchial asthma and in byssinosis. In general, decreased flow rates reflect airway constriction, while increased flows indicate dilatation of small airways.[11] The method lends itself to controlled trials with drugs in which each subject serves as his own control, as in the present study.

With this technique to assess airway caliber changes after histamine inhalation, we have shown that a single oral dose of 500 mg. ascorbic acid inhibits the constrictor effect of histamine on airways of human subjects in vivo. This effect lasts at least 6 hours.

Reductions of expiratory flow rates accompany the acute symptoms of byssinosis in textile workers. Recently, Valic and Zuskin (unpublished material) demonstrated that ascorbic acid (500 mg. orally) prevented this effect of exposure to textile dusts to a large extent. The acute constrictor effect of textile dust in man is caused, at least in part, by the presence of a water-soluble histamine-releasing agent in these dusts.[12, 13] The action of ascorbic acid in preventing the acute effect of dust exposure in textile workers is thus consistent with the results of the present study. Both actions can be explained by an antagonistic action of ascorbic acid against the effect of histamine on human airway smooth muscle.

Experiments with anesthetized guinea pigs[3] suggested that ascorbic acid has a direct relaxant effect on airway smooth muscle, and our results confirm this (Fig. 4, *A*). In the guinea pig, ascorbic acid antagonized not only histamine, but also 5-hydroxytryptamine and bradykinin.[3] Thus, ascorbic acid does not appear to have a specific antihistamine action on airway smooth muscle.

The evidence on a possible role of β-adrenoreceptors in the action of ascorbic acid is to some extent contradictory. Dawson and West[3] found that pronethalol did not alter the inhibitory effect of ascorbic acid on airway constrictor responses in vivo. In the present study, propranolol reduced the relaxant effect of ascorbic acid on the guinea pig trachea in vitro. On the other hand, the experiments with human subjects (Table III) suggest that ascorbic acid can protect against histamine-induced airway constriction even in the presence of β-receptor blockade induced by propranolol. There may be species differences in the mechanism of action, or it may be that the dose of ascorbic acid used in man (500 mg.) is sufficient to overcome the competitive block of β-receptors by propranolol.

A large dose of ascorbic acid is excreted for the most part in about 4 to 5 hours.[14, 15] To maintain the desired effect on airway smooth muscle, it may be useful to administer smaller doses (i.e., 250 mg.) at 3 hour intervals. Such a dosage schedule might be of use in preventing the acute symptoms of byssinosis in textile workers, and may also be of interest for the treatment of bronchial asthma. Since ascorbic acid in these doses has no important side effects,[16] controlled clinical trials to test the efficacy of this drug in bronchial asthma and in byssinosis may be in order.

The decrease of control flow rates at 3 and 6 hours (Table I) is intriguing and cannot be explained adequately by circadian variation (Table II). It seems more likely that the decrease is caused by a residual effect of the previous histamine inhalation experiments. Histamine is a short-acting drug, and its direct action on airways lasts at most 30 to 45 minutes,[17] but restitution of airways to control conditions may take longer.

It should be emphasized that the present experiments concern doses of histamine aerosols that can safely be given to healthy subjects since they elicit at most slight dyspnea and wheezing. The use of a sensitive method of assessing the functional effects of histamine enabled us to obtain significant results with the doses employed. We have not investigated the action of ascorbic acid against more severe degrees of histamine-induced airway constriction. Thus, at present the possible therapeutic implications of our study are limited to mild degrees of bronchial asthma.

Recently, Pauling[18] suggested that man requires 2.0 to 2.6 Gm. ascorbic acid daily for optimal health. It is conceivable that minimal airway smooth muscle tone is one aspect of optimal health, but our study was not designed to test this hypothesis. Pauling has also summarized controlled trials that suggest a protective action of ascorbic acid against clinical effects of common cold viruses.[19] The inhibition of airway constriction by ascorbic acid, which we demonstrate in the present paper, might have a beneficial effect in the common cold, but unfortunately little is known about the functional aspects of acute airway responses in these viral diseases. The study of their therapy might be facilitated by inclusion of objective lung function tests, such as flow-volume curves, in the protocol of clinical trials.

REFERENCES

1 Reisseissen, F. D.: De pulmonis structura, Strasbourg, 1803.
2 Cited by H. Oreckin-Duchemin: Documents sur la vie et l'oeuvre de F. D. Reisseissen (1773-1828), Université de Strasbourg, Faculté de Médecine, 1971.
3 Dawson, W., and West, G. B.: The nature of the antagonism of bronchospasm in the guinea-pig by ascorbic acid, J. Pharm. Pharmacol. 17: 595, 1965.
4 Hagiesco, D., Bazavan, G., Criscota, M., and Cioranesco, M.: Essais de traitement de l'asthme pulmonaire par l'acide ascorbique lévogyre (vitamine C), Presse méd. 46: 1435, 1938; J. A. M. A. 111: 1885, 1938 (abst.).
5 Hunt, H. B.: Ascorbic acid in bronchial asthma. Report of a therapeutic trial on twenty-five cases, Br. Med. J. 1: 726, 1938.
6 Dautrebande, L.: Experimental observations on the participation of alveolar spaces in airway dynamics, *in* Bouhuys, A., editor: Airway dynamics, Springfield, Ill., 1970, Charles C Thomas, Publisher, p. 153.

7 Bouhuys, A., Hunt, V. R., Kim, B. M., and Zapletal, A.: Maximum expiratory flow rates in induced bronchoconstriction in man, J. Clin. Invest. 48: 1159, 1969.
8 Constantine, J. W.: The spirally cut tracheal strip preparation, J. Pharm. Pharmacol. 17: 384, 1965.
9 Turner, P., Burman, J., Hicks, D. C., Cherrington, N. K., MacKinnon, J., Waller, T., and Woolnough, M.: A comparison of the effects of propranolol and practolol on forced expiratory volume and resting heart rate in normal subjects, Arch. Int. Pharmacodyn. Ther. 191: 104, 1971.
10 Shand, D. G., Nuckolls, E. M., and Oates, J. A.: Plasma propranolol levels in adults with observations in four children, CLIN. PHARMACOL. THER. 11: 112, 1970.
11 Bouhuys, A.: Airway dynamics and bronchoconstrictive agents in man, *in* Bouhuys, A., editor: Airway dynamics, Springfield, Ill., 1970, Charles C Thomas, Publisher, p. 263.
12 Bouhuys, A., and Lindell, S. E.: Release of histamine by cotton dust extracts from human lung tissue in vitro, Experientia 17: 211, 1961.
13 Nicholls, P. J., Nicholls, G. R., and Bouhuys, A.: Histamine release by compound 48/80 and textile dusts from lung tissue in vitro, Inhaled particles and vapours, Oxford, 1966, Pergamon Press, Inc., p. 69.
14 Wright, I., Lilienfeld, A., and MacLenathen, E.: Determination of vitamin C saturation, Arch. Intern. Med. 60: 264, 1937.
15 Grollman, A.: Pharmacology and therapeutics, ed. 4, Philadelphia, 1960, Lea & Febiger, p. 879.
16 Goodman, L. S., and Gilman, A.: The pharmacological basis of therapeutics, ed. 3, New York, 1965, The MacMillan Co., p. 1665.
17 Bouhuys, A., Jonsson, R., Lichtneckert, S., Lindell, S. E., Lundgren, C., Lundin, C., and Ringquist, T. R.: Effects of histamine on pulmonary ventilation in man, Clin. Sci. 19: 79, 1960.
18 Pauling, L.: Evolution and the need for ascorbic acid, Proc. Nat. Acad. Sci. 67: 1643, 1970.
19 Pauling, L.: The significance of the evidence about ascorbic acid and the common cold, Proc. Nat. Acad. Sci. 68: 2678, 1971.

Chinese Medical Journal, 66: 605-608, November, (1948).

THE ASCORBIC ACID (VITAMIN C) REQUIREMENT FOR TISSUE SATURATION IN CHINESE COLLEGE STUDENTS

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A number of studies have been reported in countries on the human requirement of ascorbic acid, but few such studies have been made in China. Chen and co-workers (*1*) studied the ascorbic acid requirements of Chinese scurvy patients. Wang (*2*) made similar studies on four normal Chinese adults and arrived at the rather low figure of 37.1 mg. per person per day, or 0.75 mg. per Kg. body weight.

EXPERIMENTAL

Subjects:

Four healthy Chinese students, 2 men and 2 women, served as subjects; they are described in Table I. The subjects were engaged in normal school activities, and vigorous physical exertion was avoided during the period of experiment.

TABLE I. EXPERIMENTAL SUBJECTS

Subjects	*Sex*	*Age*	*Wt.*	*Ht.*
Y. P. F.	male	28	53 Kg.	169 cm.
S. S. C.	male	24	64 Kg.	174 cm.
C. T. Y.	female	25	50 Kg.	154 cm.
W. C. C.	female	30	56 Kg.	158 cm.

Basal Diet:

The basal diet was adequate in all respects except ascorbic acid, and consisted of food commonly consumed by middle class Chinese. The diet was calculated to provide 2,600-2,800 calories, 83 Gm. of protein, 0.72 Gm. of Ca, 1.04 Gm. of P, and 30 mg. of Fe per person per day. No tea was allowed during the experiment. The foods were prepared under strict supervision in the experimental kitchen of the Home Economics Department and special care was taken to cook all food thoroughly in order to insure the maximum destruction of ascorbic acid. The subjects showed good appetites and either maintained their body weights or showed slight gains in weight during the course of the experiment. Table II shows the character of the basal diet. The amount of ascorbic acid supplied by this diet, as determined by chemical analysis at weekly intervals, averaged 5.0 mg. per day.

Collection of Urine and Methods of Analysis:

Complete 24-hour urinary specimens were collected in glass bottles which contained 50 ml. of 2% metaphosphoric acid in 5N H_2SO_4. The experiment was performed during February, March and April of 1946 at Chengtu at which time the temperature fluctuated between 12 and 14°C. Repeated tests on the loss of ascorbic acid in the urine preserved under these conditions showed an average loss of 8%, a figure similar to that of Todhunter and Robbins (*6*) who reported a loss of 5% in 24 hours. When known amounts of ascorbic acid were added to the urine, 103 to 105% was recovered.

The ascorbic acid content of the food and urine was determined by titration with 2, 6-dichlorophenolindolephenol. The food samples were triturated with a solu-

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Received for publication September 18, 1947.

TABLE II. AVERAGE FOOD CONSUMPTION PER CAPITA PER DAY ON AN ASCORBIC ACID DEFICIENT DIET

(figures in grams)

Breakfast		*Lunch*		*Supper*	
Food	Amount	Food	Amount	Food	Amount
Steamed Bread	75	Rice*	100	Rice*	100
		Mustard Root	50		
Soft Rice*	60			Pork	60
		Starch Noodles	15		
Wheat Bran	20	Dried Fungus	5	Mustard Root	100
		Dried Yellow Lily	5		
Eggs	50	Carrots	70	Carrots	30
Roasted Peanuts	20	Bean Curd (dried)	70	Chinese Lettuce	30
Salted Turnip	30	Salted Turnip	30	Oil	60
		Chinese Lettuce Root	100		
				Sugar	10
		Pork	60		

* Reckoned on a dry basis

tion consisting of equal volumes of 3% metaphosphoric acid and 3% trichloracetic acid and fine, acid-washed sand. The mixture was centrifuged and aliquots of the clear supernatant liquid were titrated with the dye solution. The dye solution was standardized with freshly prepared crystalline ascorbic acid solution each time just before using. Analyses were repeated until triplicate checked readings were obtained. In many cases, the determinations were carried out by two separate individuals.

Plan of Experiment:

The general plan of the experiment followed that of Belser, Hauck and Storvick (*3*) with slight modifications. The subjects were first saturated with ascorbic acid by adding to the regular dormitory diet for 3 to 5 days either 4 to 6 oranges or 200 mg. of ascorbic acid daily. At the end of this saturation period, the subjects were placed on the basal diet and given orally a test dose of 500 mg. of ascorbic acid. The response was measured in terms of the urinary excretion of ascorbic acid in the following 24 hours. Three such saturation periods were included in the experiment. The first saturation period was placed at the beginning of the experiment at which time the subjects were taking 4 to 6 oranges a day for 3 days and 200 mg. of ascorbic acid for two more days. The second saturation period followed when the whole study was about one third complete. The third saturation period took place at the end of the study. Subject C. T. Y. started on the experiment two days later than the others; therefore only two saturation periods were arranged for this subject. The lowest ascorbic acid excretion after administering the test dose was taken to indicate the saturation level for each subject. Thus each subject showed a different saturation standard.

After saturation was attained, crystalline ascorbic acid was administered orally at gradually increasing levels. For each period the subjects were placed on the given level of intake for 6 days and on the 7th day, 500 mg. of test dose was given orally at 1 P.M. and the urinary excretion of ascorbic acid for the next 24 hours was determined. When the test dose response (ascorbic acid excretion) for any subject on a given intake of ascorbic acid equaled or just exceeded his own saturation standard, the ascorbic acid intake for that period was considered the amount needed to produce saturation in that subject.

RESULTS

The results of the experiment are summarized in Table III.

A total of 7 experimental periods in addition to the 3 saturation periods were included in this study. The level of intake was increased from 15 mg. per day until it reached 115 mg. The experiment lasted for 60 days during which time the diets of all the subjects were under rigid control.

TABLE III. 24 HOUR URINARY EXCRETION OF ASCORBIC ACID IN RESPONSE TO 500-MG. TEST DOSE AT DIFFERENT LEVELS OF ASCORBIC ACID INTAKE

(figures in milligrams)

Periods	*Ascorbic Acid Intake**	*No. of Days on Experiment*	*Subjects*			
			Y.P.F.	*S.S.C.*	*W.C.C.*	*C.T.Y.*
Saturation Periods I	**	5	411	400	385	—
II	205	3	352	299	304	338
III	205	3	342	327	415	396
Experimental Periods I	15	6	171	177	206	230
II	30	6	215	261	103	316
III	45	6	258	246	260	257
IV	60	6	246	245	269	307
V	75	6	277	306	305	267
VI	90	6	303	298	—	336
VII	115	6	313	346	—	396

* including 5.0 mg. of ascorbic acid in basal diet.

** For the first 3 days, the subjects consumed 4 to 6 oranges daily, then following this 200 mg. ascorbic daily for 2 days. Subject CTY took 6 oranges per day for 3 days only.

The saturation standard for each subject as determined by the aforementioned technique were as follows: Y.P.F., 342. mg.; S.S.C., 299 mg.; W.C.C., 304 mg.; and C.T.Y., 338 mg. Individual variations observed were similar to those reported by other workers (*3,6*).

When the levels of ascorbic acid intake were gradually increased, subjects Y.P.F. and S.S.C. showed a regular step-wise increase in urinary excretion in response to the 500-mg. test dose. The response of the other two subjects was less regular. The sudden drop of W.C.C. at the 30-mg. level might have been related to mental strain when the subject was engaged in intensive research work.

From Table III, it may be noted that the minimum amount of ascorbic acid intake per day necessary to saturate Y.P.F. was 115 mg.; C.T.Y., 90 mg.; S.S.C. and W.C.C. 75 mg. The average saturation value for the 4 subjects was therefore 89 mg. Calculated on a body weight basis, the saturation values for the different subjects were: Y.P.F., 2.17 mg.; C.T.Y. 1.80 mg.; S.S.C., 1.17 mg. and W.C.C., 1.34 mg. with an average of 1.62 mg. per Kg.

DISCUSSION

The level of ascorbic acid intake necessary for tissue saturation in human subjects as reported in the literature varies considerably according to the technique used. Van Eekelen (*4*) considered that a state of saturation exists when the daily urinary excretion of ascorbic acid shows a distinct rise above the basal level. He reported that the requirement of ascorbic acid for a 70-Kg. person is 60 mg. Harris (*5*) regarded a subject as saturated when, after a series of daily test doses of 700 mg. of ascorbic acid per 10 stone (or 140 lb.) body weight, the 2¼-hour uninary sample, collected 3½ hours after the oral administration of the test dose, shows a definite increase above the basal level. His results show that a state of saturation may be obtained with an intake of 25 to 50 mg. per day. American workers report higher figures. Belser, Hauck and Storvick (*3*) found that the requirement of ascorbic acid for tissue saturation was 75 to 110 mg. per person per day, or 1.0 to 1.6 mg. per Kg. Todhunter and Robbins (*6*) noted the amount of ascorbic acid required to saturate 3 normal women as 80 to 110 mg. per person per day, or 1.6 to 1.7 mg. per Kg. Fincke and Lanquist (*7*) reported a slightly higher value, 111 to 131 mg. per person per day, or 1.6 to 1.7 mg. per Kg. These figures are in general agreement with the results of the present study. It would appear that racial traits and dietary habits do not affect the ascorbic acid requirement.

SUMMARY

The average ascorbic acid requirement for tissue saturation in four normal Chinese college students is 89 mg. per person per day, or 1.62 mg. per Kg. body weight. These values correspond to the figures reported by American workers.

Acknowledgment: The authors wish to express their gratitude to Prof. C. Tsai of National Central University, to Prof. J. C. Thomson of the University of Nanking and to Prof. C. Y. Lin of West China Union University for providing the chemicals necessary for this experiment.

REFERENCES

1. CHEN, K. C., YU, T. F., LIU, S. H., and CHU, H. I.: *Chinese J. Physiol.*, **15**: 119, 1939.
2. WANG, C. F.: *J. Chinese Chem. Soc.*, **9**: 97, 1939.
3. BELSER, W. B., HAUCK, H. M., and STORVICK, C. A.: *J. Nutrition*, **17**: 513, 1939.
4. VAN EEKELEN, M.: *Biochem. J.*, **30**: 2291, 1936.
5. HARRIS, L. H.: *The Lancet*, 642, May 30, 1942.
6. TODHUNTER, E. N., and ROBBINS, R. C.: *J. Nutrition*, **19**: 263, 1940.
7. FINCKE, M. L., and LANDQUIST, V. L.: *J. Nutrition*, **23**: 483, 1942.

Anal. Chim. Acta 56(2): 325-328 9462

Analytica Chimica Acta
Elsevier Publishing Company, Amsterdam
Printed in The Netherlands

Titrimetric determination of ascorbic acid with cerium(IV) sulphate

Sullman[1] appears to have been the first to use cerium(IV) sulphate for the determination of ascorbic acid. Treating an aliquot with an excess of cerium(IV) sulphate solution and back-titrating after about 30 min with standard iron(II) solution, he reported that one mole of ascorbic acid consumed an amount of cerium(IV) equivalent to three oxygen atoms; the products were believed to be 1-threonic acid and carbon dioxide. Repeating the work of Sullman, Gopala Rao and Narayana Rao (unpublished work, 1957) found that the oxidation of ascorbic acid by excess of cerium(IV) does not correspond to any definite oxidation stage but is largely influenced by the time of contact of reactants, their relative ratio, the acidity of the medium and the temperature. Rao and Somidevamma (unpublished work, 1953) attempted the direct titration of ascorbic acid with cerium(IV) sulphate in dilute sulphuric acid medium using redox indicators. With diphenylbenzidine indicator, the error was observed to be +0.6–+1.0%. With ferroin, N-phenylanthranilic acid and the fluorescent indicator, rhodamine 6G, the consumption of cerium(IV) was 2-3% more than that corresponding to the oxidation of ascorbic acid to dehydroascorbic acid. Evidently the excess consumption of cerium(IV) is due to the further oxidation of dehydroascorbic acid.

It has now been observed that ferriin, the oxidised product of ferroin, is reduced by both ascorbic acid and dehydroascorbic acid in 0.1–0.5 *M* sulphuric acid. The speed of reduction of ferriin by dehydroascorbic acid decreases as the acid concentration is increased, but the speed of reduction of ferriin by ascorbic acid is not affected by increased acidity even upto 1.5 *M*. The addition of 1 ml of syrupy orthophosphoric acid in a volume of 50 ml inhibits the reduction of ferriin by dehydroascorbic acid in a medium containing 0.75–1.25 *M* sulphuric acid but not that of ferriin by ascorbic acid. This concentration of phosphoric acid does not affect the speed of oxidation of ferroin by cerium(IV). These observations indicate that a direct titration of ascorbic acid with cerium(IV) sulphate can be made in 0.75–1.25 *M* sulphuric acid medium, without interference from dehydroascorbic acid, if ferroin is used as indicator and phosphoric acid is added. Phosphoric acid may also retard the direct reaction between ascorbic acid and cerium(IV) because of complexation with the latter. Suitable conditions for the accurate determination of ascorbic acid with cerium(IV) on this basis are described below. Ascorbic acid is oxidized stoichiometrically to the dehydroascorbic acid stage.

Experimental

Preparation of solutions. A 0.1 *M* solution of cerium(IV) in 0.5 *M* sulphuric acid medium was prepared from cerium(III) oxalate by the classical method of Willard and Young and standardised against "AnalaR" sodium oxalate.

An aqueous 0.05 *M* solution of ascorbic acid (B.P. grade, Hoffmann-La Roche) was standardised daily against potassium iodate (p.a., E. Merck) as described by Ballentine[2].

The orthophosphoric acid used was guaranteed reagent (E. Merck) and all other chemicals were of analytical-reagent quality. All solutions were made with

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deionized water.

Recommended procedure. Treat 5–20 ml of 0.05 *M* ascorbic acid with enough 2 *M* sulphuric acid to give a final acidity of 0.75–1.25 *M* on dilution to 50 ml. If the acidity falls below 0.5 *M* during the titration, cerium(IV) phosphate may precipitate. Add 1 ml of syrupy orthophosphoric acid and 1 drop of 0.01 *M* ferroin sulphate solution. Titrate with 0.1 *M* cerium(IV) sulphate solution, at normal speed with constant stirring; if any cerium(IV) phosphate precipitates, it dissolves on stirring. Near the equivalence point, add the titrant in fractions of a drop, with the aid of a thin glass rod.

The indicator correction is negligible for 0.05–0.1 *M* cerium(IV) solutions. The colour transition at the end-point is red to pale blue.

Under the above conditions, barium diphenylamine sulphonate and N-phenyl anthranilic acid can also be used satisfactorily as redox indicators, the colour transitions being from colourless to violet or red-violet. Rhodamine 6G can also be used successfully; the addition of 0.1 ml of 0.035% Rhodamine 6G solution imparts in daylight a greenish fluorescence, which is sharply quenched by a slight excess of cerium(IV).

Results and discussion

Representative results from a large number of titrations are presented in Table I.

TABLE I

TITRIMETRIC DETERMINATION OF ASCORBIC ACID WITH CERIUM(IV) SULPHATE

Indicator	*Amount of ascorbic acid (mg)*		*Indicator*	*Amount of ascorbic acid (mg)*	
	Taken	*Found*		*Taken*	*Found*
Ferroin	17.33	17.28	N-Phenylanthranilic acid	15.11	15.11
	37.09	37.13		29.88	29.83
	52.00	51.95		59.75	59.85
	66.73	66.82		72.16	72.06
Barium diphenylamine sulphonate	12.67	12.67	Rhodamine 6G	24.66	24.66
	31.00	31.06		39.63	39.72
	62.36	62.36		57.12	57.19
	84.14	83.79		72.06	71.90

Interferences. No interferences were found from 10-fold amounts of citric acid, tartaric acid, glucose, fructose or sucrose. Oxalic acid caused an excess consumption of 0.9%–2.5% of cerium(IV), when the amount of oxalic acid added varied from 0.5 to 5 times that of the ascorbic acid taken.

Titration of 0.01–0.005 *M solutions of ascorbic acid.* In the titration of such dilute solutions with ferroin as indicator, low results were obtained. This was traced to loss of ascorbic acid by autooxidation catalysed by cerium(III). Titrations made in a carbon dioxide atmosphere gave good results.

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Titrations in phosphoric acid mesium. It is unnecessary to maintain an inert atmosphere even for these dilute solutions of ascorbic acid, if the titration is done in 12–13 M phosphoric acid medium with ferroin as indicator. The Ce(IV)/Ce(III) potential in such a medium is reported to be 1.22 V[3], the value in 1–2 N sulphuric acid being 1.44 V. It was also found that the formal potentials of the dehydroascorbic acid/ascorbic acid system increase as the phosphoric acid concentration increases; this increased potential affords protection to the ascorbic acid against atmospheric oxidation. Moreover, phosphoric acid complexes any heavy metal ions present, thus preventing their catalytic action on the oxidation of ascorbic acid. The results obtained for small amounts of ascorbic acid by titration in 1 M sulphuric acid medium under an inert atmosphere, and in 12–13 M phosphoric acid medium without an inert atmosphere, agreed between themselves and with the theoretical value within $\pm 0.1\%$.

A further advantage with phosphoric acid as the titration medium is that oxalic acid does not interfere as it does in sulphuric acid medium. The relative error in the determination of ascorbic acid by potentiometric titration with cerium(IV) sulphate in 12–13 M phosphoric acid medium is within $\pm 0.1\%$. In this connection, it is interesting to note that the potentiometric titration of ascorbic acid with dichlorophenolindophenol has been reported as unsatisfactory by Kirk and Tressler[4], and that with iodine as inaccurate by Stevens[5].

TABLE II

FORMAL POTENTIALS IN PHOSPHORIC ACID MEDIA

Concentration of phosphoric acid (M)	*Formal redox potential (V)*
1.0	0.445
2.0	0.464
4.0	0.506
6.0	0.568
8.0	0.609
10.0	0.650
12.0	0.686

Phosphoric acid affords a third advantage as a titration medium. From a large number of experiments, it was observed that potentiometric titration of even high concentrations of ascorbic acid with cerium(IV) sulphate in 0.75–1.0 M sulphuric acid medium (even with the addition of 1.0 ml of syrupy phosphoric acid per 50 ml) gave results about 10% lower than the theoretical. Accurate titres were secured by carrying out the potentiometric titration in 12–13 M phosphoric acid, with a bright platinum wire as indicator electrode and a saturated calomel reference electrode; initially the potentials were measured immediately after the addition of the cerium(IV) reagent, and at 2-min intervals near the equivalence points. The potential break at the equivalence point is about 170–180 mV per 0.04 ml of the reagent. The cerium(IV) reagent is taken at double the concentration of the ascorbic acid so that the phosphoric acid concentration in the titration medium is not unduly lowered.

Formal redox potentials of the dehydroascorbic acid/ascorbic acid system at varying phosphoric acid concentrations are presented in Table II.

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1 H. SULLMAN, *Enzymologia*, 5 (1938) 326.
2 R. BALLENTINE, *Ind. Eng. Chem., Anal. Ed.*, 13 (1941) 89.
3 G. GOPALA RAO, P. KANTA RAO AND S. BHANOJEE RAO, *Talanta*, 11 (1964) 825.
4 M. M. KIRK AND D. K. TRESSLER, *Ind. Eng. Chem.*, 11 (1939) 322.
5 J. W. STEVENS, *Ind. Eng. Chem.*, 10 (1938) 269.